THE EFFECTS OF SYNTHETIC ESTROGEN ON SEXUAL SELECTION AND HEPATIC GENE EXPRESSION PATTERNS IN THE SEX-ROLE-REVERSED GULF PIPEFISH

A Dissertation

by

EMILY ROSE

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Chair of Committee,	Adam Gregory Jones
Committee Members,	Gil Rosenthal
	Miguel Mora
	Daniel Roelke
Head of Department,	Thomas McKnight

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ABSTRACT

Species in which sexual selection acts more strongly on females than on males provide interesting opportunities to study the evolution of sex roles and sex differences and the impact of exposure to endocrine disruptors. The sex-role-reversed Gulf pipefish (*Syngnathus scovelli*) is sexually dimorphic, and females possess dramatic secondary sexual traits while males are choosy. Experiments involving endocrine disruptors have previously illustrated that the secondary sexual traits in female pipefish are estrogen-regulated, but no studies before this dissertation had yet addressed the effects of endocrine disruptors on the strength of sexual selection or the hormonal regulation of a sexually dimorphic transcriptome in any sex-role-reversed taxon. When exposed to low, ecologically relevant concentrations of 17α-ethinylestradiol (EE2) of 2 ng/L, female pipefish had greater reproductive successes which resulted in an increase in sexual selection acting on exposed females. However, at minimally higher and still ecologically-relevant EE2 concentrations (5ng/L), male pipefish were unable to receive eggs or maintain their pregnancies, ceasing the reproduction of the exposed fishes. Understanding the evolutionary consequences of exposure to endocrine disruptors at several concentrations is important to help predict the effects that endocrine disruptors will have on the fecundity and sustainability of exposed natural populations.

Using next-generation RNA-sequencing technology, I compared gene expression patterns in the livers of pipefish females, pregnant males, and non-pregnant males exposed to EE2 at ecologically relevant concentrations of 5ng/L. The results showed that the control Gulf pipefish liver transcriptomes showed sexually dimorphic

patterns of gene expression, indicating that this sex-role-reversed pipefish species does not appear to have an endocrine reversal from the typical gene expression patterns found in the livers of most fishes with conventional sex roles. Estrogen exposure did cause feminization of gene expression patterns in the male liver transcriptome, with several of the EE2 responsive genes shown to have female-biased expression in control animals. These genes included several of the classic estrogen biomarkers, such as *vitellogenin*, *choriogenin*, and *zona pellucida* transcripts. Overall, exposure to synthetic estrogen has been shown to have a strong effect on selection, reproductive abilities, and hepatic gene expression levels in the sex-role-reversed Gulf pipefish.

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1. INTRODUCTION

The two primary goals for this dissertation were to first identify the roles of pre- and post-copulatory episodes of selection and secondly, determine the effects an endocrine disruptor synthetic estrogen, on the mating system, gene expression patterns, and on selection in a sex-role reversed fish, the Gulf pipefish (*Syngnanthus scovelli*). Before I could determine the effects of an endocrine disruptor, such as 17α ethinylestradiol (EE2), on a sex-role reversed mating system, I first had to determine the strength of both natural and sexual selection on the system. The second section of my dissertation includes a study where I broke down total selection into pre- and post-copulatory phases to calculate the contributions that each episode of natural and sexual selection had and their interactions. Once I understood the breakdown of total selection, I next could ask the question of how does synthetic estrogen affect the Gulf pipefish's mating system in regards to reproductive success, mating success, and brood survivorship in my third section of my dissertation.

The next question I chose to address in my fourth section of my dissertation was how synthetic estrogen affects gene expression patterns in the livers of male and female pipefish, as well as both pregnant and non-pregnant males. To address this question, I used next-generation sequencing technology to sequence the entire liver transcriptomes of Gulf pipefish and compare RNA expression patterns in livers of either control fish or fish exposed to 5ng/L EE2 for seven days. My transcriptomics study also allowed me to test for sexually dimorphic genes expression patterns in a sex-role reversed, which had never been done before. My fifth and last section of my dissertation includes a study

where I developed new microsatellite markers to confirm a genetic monogamous mating system in the dwarf seahorse, *Hippocampus zosterae*.

2. THE CONTRIBUTIONS OF PREMATING AND POSTMATING SELECTION EPISODES TO TOTAL SELECTION IN SEX-ROLE-REVERSED GULF PIPEFISH*

Introduction

Beginning with Charles Darwin's first description of sexual selection (Darwin 1859; Darwin 1871), theoretical and empirical studies in this arena have focused largely on how events occurring before mating, such as female choice and male-male competition, affect reproductive success (Andersson 1994; Andersson and Simmons 2006; Jones and Ratterman 2009). Over the last several decades, research on mating competition has been linked with formal selection theory to produce a powerful set of inferential tools for the study of precopulatory sexual selection of the type Darwin originally described (Wade 1979; Wade and Arnold 1980; Arnold and Wade 1984*a*, 1984*b*; Hamon 2005; Shuster 2009). However, precopulatory mechanisms of mate choice are only part of the sexual selection story. A vast array of behavioral, morphological, and physiological traits can affect reproductive success during and after mating, and a major research enterprise has emerged and grown since the 1970s to focus attention on the fitness effects of these postmating processes (Parker 1970; Thornhill 1983; Birkhead and Pizzari 2002; Eberhard 2009). In recent years, sperm competition, cryptic female choice, and sexual conflict have taken center stage in the study of

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postmating processes. Any one of these mechanisms has the potential to reinforce or counteract the effects of precopulatory sexual selection. In addition, other forms of natural selection, such as fertility selection or offspring viability, have the potential to act in the same direction or in opposition to precopulatory sexual selection. Consequently, sexual selection is a complex process that does not stop at mating, as Darwin seemed to imply in his original treatise (Darwin 1871). To fully understand the total strength of selection acting on a sexually selected trait, we need to understand the mechanisms producing premating and postmating selection, including postmating natural and sexual selection, and the interactions between premating and postmating processes.

Typically, constraints imposed by experimental systems require premating and postmating events to be studied separately. However, the goal of elucidating potentially important tradeoffs between premating and postmating phases of selection will require studies that measure the effects of both processes in the same individuals within a single experiment. In water striders (*Gerris lacustris*), premating and postmating mechanisms have been found to act antagonistically (Danielsson 2001), whereas in western rainbow fish (*Melanotaenia australis),* they act concordantly (Young et al. 2010). Although these studies surveyed both episodes of sexual selection, they were unable to quantify the relative contributions of each episode due to empirical constraints, (e.g. limited sample sizes, an inability to assign total male fitness, or a failure to quantify selection coefficients). Thus, in many systems we are aware that both premating and postmating processes occur, and we may even know whether they reinforce or oppose one another,

but we seldom know the relative magnitudes of the different phases of selection (Andersson and Simmons 2006; Hunt et al. 2009).

The theoretical and methodological framework necessary to divide total selection into its component parts exists (Arnold and Wade 1984*a*, 1984*b*), and its widespread application to empirical systems could contribute to a better understanding of the importance of premating and postmating natural and sexual selection in secondary sexual trait evolution. Three recent papers have adopted approaches related to those described by Arnold and Wade (1984a, 1984b) to decompose selection coefficients or variances in fitness into parts arising before and after mating. First, Pischedda and Rice (2012) conducted a study of *Drosophila melanogaster* in which they compared the variances in fitness arising from precopulatory and postcopulatory sexual selection. They found mating success and male fertilization success to be equivalent contributors toward total sexual selection, but after adjusting for male mating order, the postcopulatory episode was shown to be much less important than the precopulatory episode. The second study, conducted by Droge-Young and colleagues (2012), focused on the covariance among selection episodes in *Drosophila melanogaster* and found no significant relationships between pre- and postcopulatory selection. They did, however, detect a positive relationship between sperm competitive success and offspring viability, suggesting that this component of natural selection acts in the same direction as postcopulatory sexual selection. Finally, Collet *et al*. (2012) found patterns of multiple paternity in semi-natural groups of red junglefowl, *Gallus gallus*, to be correlated with the strength of sexual selection episodes. In replicate groups that exhibited low levels of

multiple mating by females, male status, a target of precopulatory processes, was strongly selected. However, in groups with high levels of multiple mating by females, there was little variation in the mating success of males, resulting in a stronger role for postcopulatory mechanisms and weaker overall sexual selection. This latter study demonstrates that the reproductive ecological setting can influence the relative importance of the various sources of selection.

The Gulf pipefish (*Syngnathus scovelli*) has been characterized sufficiently from both precopulatory and postcopulatory perspectives to provide *a priori* predictions regarding the relationships among different phases of selection. The Gulf pipefish is a sexually dimorphic species found along the Gulf of Mexico coastline and Atlantic coast of Florida (Dawson 1985). As in all members of the Family Syngnathidae (pipefishes, seahorses and seadragons), this species exhibits unilateral male parental care. Male *S. scovelli* have a brood pouch on their ventral surface, which allows the males to receive eggs from the female and to carry developing offspring over the course of an approximately two-week male pregnancy. Mature females tend to be larger than males and possess permanent iridescent bars on their keeled abdomens that males lack (Jones and Avise 2001). The Gulf pipefish is sex-role reversed in that females compete to gain access to mating opportunities with males. Molecular studies have shown that Gulf pipefish females mate with multiple males during the timeframe of a male pregnancy and that males are rarely impregnated by more than one female per pregnancy (Jones and Avise 1997*a*; Jones et al. 2001).

Studies of sexual selection in Gulf pipefish show that larger females have an advantage over smaller females in both precopulatory and postcopulatory phases. A microsatellite-based study of parentage shows that field collected males mate with the largest, most ornamented females (Jones et al. 2001), and laboratory no-choice studies indicate that mating occurs more quickly when males are given large females as potential mates as opposed to small females (Paczolt and Jones 2010). Precopulatory sexual selection on female Gulf pipefish is among the strongest ever documented for females of any species (Jones et al. 2001).

Postmating phenomena are somewhat more complex and also less well understood than premating processes in Gulf pipefish. Events occurring after mating in Gulf pipefish are best understood from work on Gulf pipefish and the congeneric broad-nosed pipefish, *Syngnathus typhle*. In all studied species in the genus *Syngnathus*, males have a brief period of receptivity, after which their pouch seals irreversibly until the end of the pregnancy. Thus, even if they have an incompletely filled pouch, pregnant males cannot mate after pouch closure until they give birth. In *S. typhle*, males usually mate with multiple females per pregnancy and receive more eggs from their first mates compared to later mates (Partridge et al. 2009). Males also give birth to a number of offspring substantially smaller than the number of eggs they initially receive (Ahnesjö 1992; Partridge et al. 2009), and embryos with larger mothers tend to experience higher survivorship in the brood pouch compared to eggs from smaller females (Ahnesjö 1996; Partridge et al. 2009; Mobley et al. 2011). Thus, the data thus far indicate that some

form of postmating natural or sexual selection is acting in *S. typhle* (Sagebakken et al. 2011; Kvarnemo et al. 2011).

Gulf pipefish differ from broad-nosed pipefish in that male Gulf pipefish normally receive eggs from only one female per pregnancy, a feature that paves the way to investigate postmating selection in a slightly simpler system than that offered by *S. typhle* (Jones and Avise 1997*a*; Jones et al. 2001). Paczolt and Jones (2010) conducted an experiment spanning two pregnancies for each focal male, in which they mated each male with two small females, two large females or a combination of large and small females. Their results showed that males preferred to mate with larger females over smaller females and that the eggs of larger females were more likely to produce viable offspring at the end of the pregnancy. They further documented a tradeoff between broods, in which a male whose previous brood originated from a large female experienced a reduction in survivorship of about 12 to 14% in his subsequent brood. Thus, Gulf pipefish, like broad-nosed pipefish, display enough variance in fitness among male broods that postmating selection could be important for the evolution of secondary sexual traits in females. Importantly, the strength of postmating selection could in principle be estimated and compared to premating sexual selection by partitioning the variance in relative fitness into premating and postmating components. An examination of the total magnitude of fitness variance arising from postmating processes should permit a diagnosis of the relative importance of premating and postmating processes as well as an assessment of whether these mechanisms reinforce or oppose one another.

Our goals in the present study were to investigate the relative roles of premating and postmating selection in artificial breeding aggregations of Gulf pipefish. In particular, we used a microsatellite-based parentage analysis to quantify the opportunity for selection, which provides a measure of the maximum strength of selection that can operate on any trait, and to partition this measure into components arising from number of mates, number of eggs transferred per mating, and offspring survivorship within the male's brood pouch. We also directly quantified selection differentials on male and female body size, a trait known to be a target of sexual selection in female pipefish, and we partitioned these selection differentials into premating and postmating components.

Methods

Sexually mature female and pregnant male Gulf pipefish were collected from shallow seagrass beds in the Gulf of Mexico near Aransas Pass, Texas, using a seine net between July and October, 2010 and transported to Texas A&M University in College Station, TX. Once in the laboratory, fish received freshwater baths to remove any external parasites, were acclimated to a salinity of 26 ppt, and were group housed, separated by sex, in a flow-through system until the males gave birth. Before entering the experiment, fish were anesthetized with clove oil and marked with three visible implant fluorescent elastomer tags (VIFE; Northwest Marine Technology, Inc.) for identification using the protocol of Woods and Martin-Smith (2004). We used two colors, yellow and blue, and each fish received one of eight marking patterns, each of which consisted of three bands with a combination of at least one yellow and one blue mark to prevent mating preference based on marking colors. No mortalities occurred

during the marking process, and marks did not affect mating success as evidenced by no difference in mating success between the eight different color combinations across replicates ($F=0.911$, $df= 7$, $p= 0.506$). Photographs were taken after marking the fish on the first, eighth and fifteenth days of the experiment to document any changes in fish size or health. The sexes were separated for three days to recover from the marking procedure.

Seven independent replicates were run over the course of the experiment, involving a total of 112 pipefish. Each replicate contained eight non-pregnant males and eight sexually mature females together in a 100 liter tank for 14 days. Tanks were equipped with biological filters and 10% water changes were performed daily. During each replicate, males were checked daily for pregnancies. On day eight of a male's pregnancy, the male was sacrificed using MS222 and the eggs were dissected from the pouch. At this stage, reduced eggs that failed to develop can easily be distinguished from successfully developing embryos (Paczolt and Jones 2010). The numbers of viable embryos and reduced eggs were recorded and used to calculate embryo survivorship as the number of eggs that developed successfully divided by the total number of eggs initially transferred by the female. To assign maternity to each brood, four embryos from the anterior end of the pouch and four from the posterior end of the pouch were preserved in ethanol for DNA extraction. We extracted DNA from the embryos by using 150µl of a Chelex and Proteinase K solution (25ml millipure water, 199 µl of Proteinase K [20mg/ml] and 1.25g Chelex) per embryo. The embryos were incubated in the solution at 56 ºC for one hour followed by 100 ºC for eight minutes. All females and

non-pregnant males were sacrificed 18 days after the replicate began. After each adult fish was sacrificed, the dorsal fin was removed and preserved in ethanol for DNA extraction using the Genomic DNA Purification Kit from Gentra systems (Qiagen).

Maternity was assigned using three hypervariable dinucleotide microsatellites (*micro25.10*, *micro25.22*, and *micro22.3*) previously developed by for *S. scovelli* (Jones and Avise 1997*a*; Partridge et al. 2009). Microsatellite PCR conditions followed previous studies (Jones and Avise 1997*a*, Jones et al. 1999) and PCR products were analyzed on an Applied BioSystems 3730xl DNA Analyzer at the Cornell University Life Sciences Core Laboratories Center. Microsatellite alleles were sized with Peak Scanner software. Gulf pipefish have unambiguous paternity; our goal was to use the microsatellite data to match each embryo to its unknown mother. Maternal alleles were determined by subtracting the known paternal allele from each offspring's genotype. Each brood contained at most two maternal alleles per locus, indicating that each pregnant male had received eggs from only a single female and facilitating maternal genotype reconstruction. Each reconstructed maternal genotype matched only one of the eight females in the corresponding experimental replicate, allowing us to exclude all but one female as the mother of each brood. We assigned parentage in all broods except for those of two males whose premature deaths resulted in underdeveloped progeny from which we were unable to amplify microsatellite loci.

Standard length, the distance from the tip of the fish's snout to the end of the caudal peduncle, was measured for each fish from the photographs taken throughout the experiment using ImageJ (NIH). We calculated the absolute and standardized selection

differentials (*s* and *s*', respectively) as the covariance between standard length and relative fitness (Lande and Arnold 1983). The standardized selection differential (*s*') is in units of phenotypic standard deviations, while the absolute selection differential (*s*) is in cm. We decomposed the total selection differential into three successive, multiplicative episodes (Arnold and Wade 1984*a*), including premating selection (number of mates), followed by two episodes of postmating selection (number of eggs transferred per mating and proportion of surviving embryos). The last two episodes are averages within individuals across mates or offspring, as described by Arnold and Wade (1984*a*). We calculated the opportunity for selection as the variance in relative fitness and decomposed it into its component selection episodes following the techniques of Arnold and Wade (1984*a*, 1984*b*). We conducted the fitness decompositions separately for each tank and then obtained the means and standard errors across tanks. To determine if episodes differed significantly from one another we compared 95-percent confidence intervals, and we used JMP 9.0 for all other statistical analyses.

Results

Characterization of the Gulf pipefish mating system

Two males died during pregnancy and two other males experienced complete brood loss (i.e., none of the progeny in their pouches survived to day eight). These other males were included in our calculation of total selection on males but not in the calculation of total selection on females, because we were unable to genotype the progeny for these failed broods. We conducted additional analyses with these broods added to either the most-successful or least-successful females and found that their

omission had minor effects on our partitioning of selection. One female was removed from the analysis, because her only mate died shortly after mating. Thus, of the original 112 individuals, our dataset contained 54 surviving males and 55 females across 7 experimental replicates.

Including the two males that died, fifty-one males mated (91%) and only five males (9%) failed to mate. As expected, each mated male received eggs from only one female; within each male's brood pouch, the four anterior end embryos and the four posterior end embryos invariably had the same mother. Males that mated did not differ significantly in standard length from unmated males (mated males: 8.7cm, SE = 0.12; unmated males: 8.1cm, SE = 0.38; Wilcoxon Rank Sum Test: $n = 54$, $p = 0.1563$, two males that died excluded), but with only five unmated males we had little power to detect a difference.

The parentage analysis indicated that twenty-six females (47%) did not mate, fifteen (27%) mated with a single male and fourteen (26%) mated multiply, having either two mates (18%) or three mates (7%). The mated females were significantly larger than the non-mated females (mated: 9.9cm, SE = 0.11; unmated: 9.6cm, SE = 0.11; student's t-test: $n = 55$, $p = 0.04$). However, there was no significant difference between the size of females that mated singly versus multiply in body length (student's t-test: $n = 29$, $p = 0.63$, Single: 9.96 cm, SE 0.150, Multiple: 9.86sm, SE: 0.156) or standard body depth (student's t-test: $n = 29$, $p = 0.97$, Single: 0.47cm, SE: 0.119, Multiple: 0.48cm, SE: 0.123).

We found that 32 males received eggs from multiply mated females and 15 carried the broods of singly mated females, (excluding the two males that died and two males with complete reduction). There was no difference in latency to mate for males that mated with singly mating versus multiply mating female partners (singly: mean = 3.1 days, SE = 0.87; multiply: 3.7 days, SE = 0.59; student's t-test: $n = 47$, $p = 0.58$). However, smaller males took significantly longer to mate than larger males ($n = 47$, $r = -0.38$, $p = 0.008$). The males that had longer delays before mating also had smaller broods than the earlier mating males ($n = 47$, $r = -0.31$, $p = 0.03$), a pattern partly explained by the observation that larger males had larger broods ($n = 47$, $r = 0.38$, $p = 0.007$). Males that mated with singly mated females received significantly more eggs (student's t-test: $n = 47$, $p = 0.0118$) and also had a higher number of surviving offspring than males mated to multiply mated females (student's t-test: $n = 47$, $p = 0.0014$.). On average, males that mated with singly mated females received 34 eggs (SE = 2.5) and had 33 surviving offspring (SE = 2.5), whereas the males that mated with multiply mated females received 26 eggs (SE = 1.7) and had an average of 23 surviving offspring (SE = 1.7) (Figure 1). Males that mated with multiply mated females had a significantly higher number of reduced eggs with an average of three reduced eggs (SE = 0.5), resulting in 9% mortality of the brood, while males that mated with singly mated females lost an average of only 0.6 eggs (SE = 0.739) or 2% of the brood. Thus, males that received eggs from multiply mating females had significantly less successful broods compared to males that mated with singly mating females (Wilcoxon Rank Sum Test: $n = 47$, $p = 0.0241$).

Multiply mated females transferred an average of 59 eggs (SE = 3.0) to their mates collectively and had 53 successful offspring (SE = 3.2) with two or three mates, a figure significantly higher than that for singly mated females, who averaged 34 eggs (SE = 2.9) transferred and 33 successful offspring (SE = 3.0) (Figure 2). However, multiply mated females transferred fewer eggs per mate (25.9, SE = 2.4) than singly mated females (34.1, SE = 2.3), a statistically significant difference (student's t-test: $n = 29$, $p = 0.009$). With respect to postmating selection, multiply mated females had a significantly higher number of eggs that failed to develop compared to singly mated females (multiply mated: mean 6.43 reduced eggs, SE = 0.68; singly mated: 0.6 reduced eggs, SE = 0.659; Wilcoxon Rank Sum Test: $n = 29$, $p < 0.0001$). As a result, singly mated females had a higher percentage of their eggs survive compared to multiply mated females (98% vs. 89% brood survivorship, respectively; Figure 2).

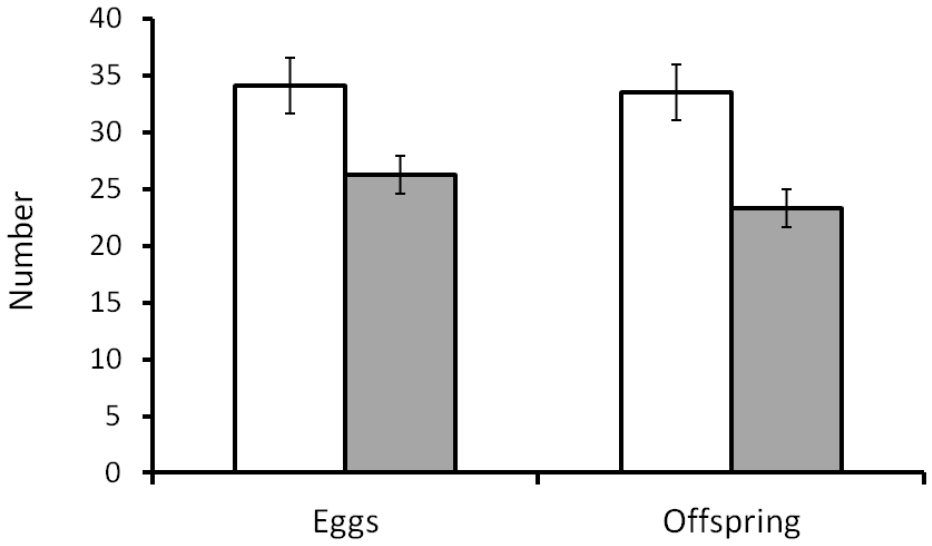

Figure 1 Total number of eggs transferred and surviving offspring in male broods originating from singly versus multiply mated female mates. The white bars represent males with singly mated female mates and the gray bars represent males with multiply mating female mates. Males that mated with singly mated females received significantly more eggs (student's t-test: $n = 47$, $p = 0.0118$) and also had a higher number of surviving offspring than males mated to multiply mated females (student's t-test: $n = 47$, $p = 0.0014$).

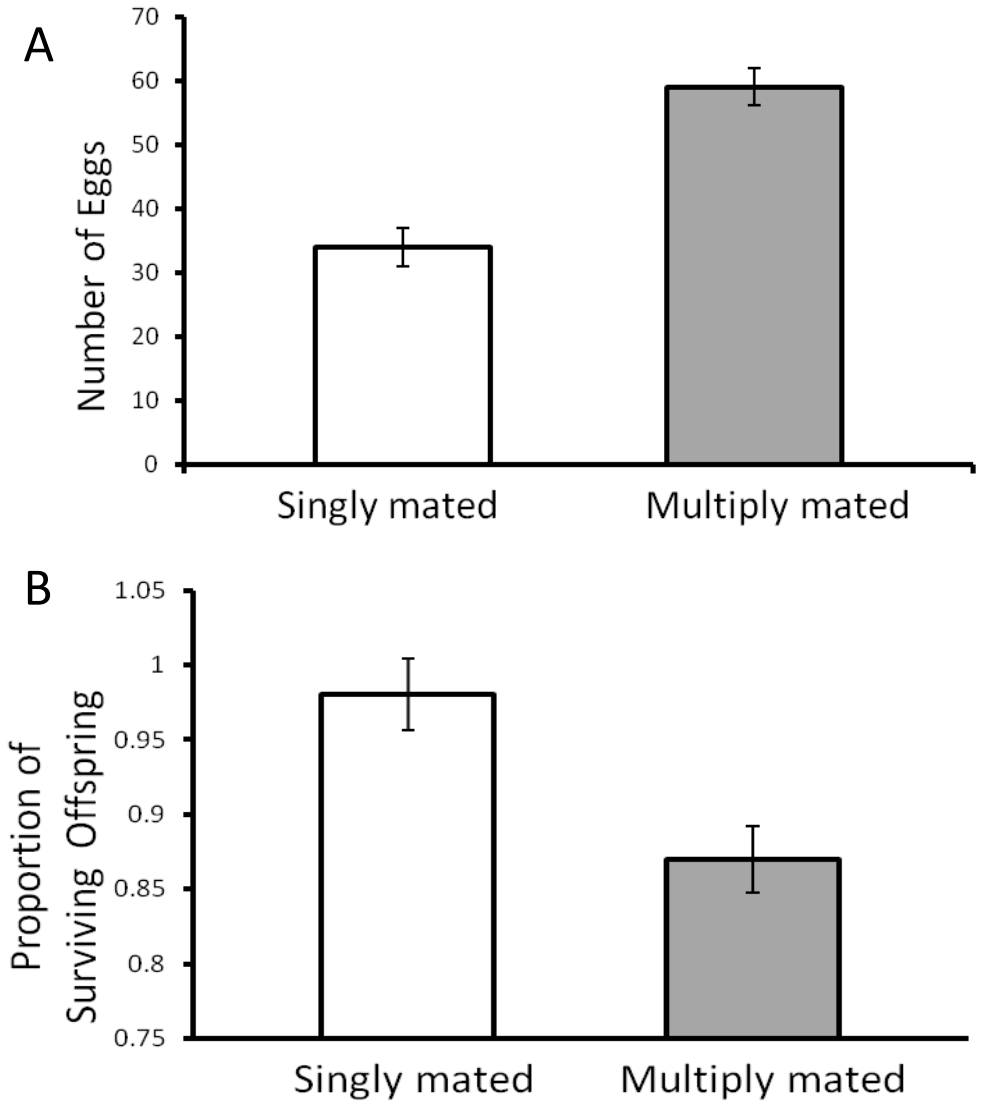

Figure 2 Total number of eggs transferred and percent survivorship of offspring originating from singly versus multiply mated females. A. Multiply mated females transferred more total eggs to their mates (summed across all of their mates) compared to singly mated females (student's t-test: $n = 29$, $p < 0.0001$). B. Singly mated females had significantly higher offspring survivorship than multiply mated females (student's t-test: $n = 29$, $p < 0.0001$). Thus, greater mating success in females resulted in lower offspring survivorship, indicating a tradeoff between pre- and postmating selection.

To determine the effects of mating order on number of eggs transferred, survivorship of consecutive broods, and size of the mates we analyzed the first two broods of multiply mated females. We included the first and second matings for 9 of the 14 multiply mated females and excluded the females that had their first two matings occur on the same day because we were not able to determine the order of the matings. We found that both the number of eggs transferred and egg survivorship were not significantly different between a female's first and second matings when compared using paired t-tests (number of eggs: $n = 9$, $p = 0.383$, SE = 3.13; egg survivorship: $n = 9$, $p = 0.210$, SE = 0.079). The first mating had an average of 23 eggs with 89% survivorship, while the second mating averaged 26 eggs with 78% survivorship. We also found no difference in the size of the male mate between the first (8.5cm) and second (8.6cm) matings, suggesting that there was no order effect on male size (paired t-test: $n = 9$, $p = 0.157$, SE = 0.322). The sample sizes for these comparisons are small so subtle differences between first and second matings for females would be difficult to detect in the present study.

Partitioning the opportunity for selection

The total opportunity for selection (I), which incorporates all three episodes of selection, was much higher in females (1.641) than in males (0.345), and this difference was significant, as evidenced by non-overlapping 95% confidence intervals (Table 1). We performed the decomposition of I separately for each replicate, and Table 1 shows means and confidence intervals across replicates. In females, the variance in fitness due to differences among individuals in mating success (I_1, also known as the opportunity for

sexual selection, I_s), was by far the most important contributor to the total opportunity for selection (Table 1). Variance in mating success was responsible for 96.4% of the variance in fitness (Table 1). The two phases of postmating selection that we measured, namely number of eggs transferred per mating and proportion of embryos surviving during the pregnancy, contributed only 4.0% and 0.6%, respectively, to the total opportunity for selection in females. Thus, in Gulf pipefish females, precopulatory sexual selection made a larger contribution to the total variance in relative fitness than did postmating mechanisms.

The decomposition of the total opportunity for selection in males showed two major sources of variance in fitness. The first was determined by whether or not a male mated, and this source of variance made up 43.2% of the opportunity for selection (Table 1). Because each mated male received eggs from only one female, there were no differences in mating success among mated males. The second major source of variance in fitness for males was the number of eggs transferred into the male's brood pouch, and this phase of selection accounted for 28.4% of the opportunity for selection on males (Table 1). Finally, males also differed in terms of the survivorship of embryos during their pregnancies, and this source of variation was responsible for 17.5% of the total variance in relative fitness (Table 1). Thus, males differed from females in that males exhibited a smaller total opportunity for selection and that the postmating phases of selection in males made larger proportional contributions to the total variance in relative fitness.

Table 1 The partitioning of the opportunity for selection in Gulf pipefish by selection episode. The most important values are shown in boldface type, and the covariance terms are shown for the sake of completeness. Further details regarding the methods of partitioning and the interpretation of the covariance terms can be found in Arnold and Wade (1984*a*, 1984*b*). Note that the covariance terms largely cancel one another out and make at most a small contribution to the overall opportunity for selection. We calculated the partitioning for each tank separately and report the means across tanks in this table. We also show 95 percent confidence intervals in brackets below each mean.

Source of Variance in Fitness	Symbol	Male Value [95% CI]	Male %	Female Value [95% CI]	Female %
Premating selection (mating success, w_1)	$\boldsymbol{I_1}$	**0.149 [-0.037, 0.335]**	**43.2%**	**1.582 [0.878, 2.287]**	**96.4%**
Postmating selection arising from number of eggs transferred (eggs per mate, w_2)	$\boldsymbol{I_2}$	**0.098 [0.049, 0.147]**	**28.4%**	**0.065 [0.022, 0.108]**	**4.0%**
Covariance between w_1 and w_2:					
Unweighted	COI(1,2)	0.105 [-0.013, 0.224]	30.5%	0.519 [0.383, 0.655]	31.6%
Weighted by number of mates	COI(1,2|1)	0.000 [0.000, 0.000]	0%	-0.064 [-0.157, 0.028]	-3.9%
Change in covariance between number of eggs (w_1w_2) and eggs per mate (w_2) caused by precopulatory sexual selection	COI(12,2|1) - COI(12,2)	-0.097 [-0.210, 0.017]	-28.0%	-0.528 [-0.651, -0.404]	-32.2%
Variance in number of eggs (w_1w_2)	**Subtotal: $\boldsymbol{I_{12}}$**	**0.256 [0.072, 0.439]**	**74.1%**	**1.574 [0.806, 2.341]**	**95.9%**
Postmating selection, embryo survivorship (embryo success, w_3)	$\boldsymbol{I_3}$	**0.060 [0.004, 0.117]**	**17.5%**	**0.009 [0.002, 0.016]**	**0.6%**
Covariance between number of eggs (w_1w_2) and embryo success (w_3):					
Unweighted	COI(12,3)	0.122 [0.000, 0.244]	35.3%	0.540 [0.406 to 0.674]	32.9%
Weighted by number of eggs	COI(12,3|2)	0.016 [-0.001, 0.033]	4.6%	0.023 [-0.032, 0.077]	1.4%
Change in covariance between total fitness ($w_1w_2w_3$) and embryo success (w_3) caused by first two episodes of selection	COI(123,3|2) - COI(123,3)	-0.109 [-0.218, 0.000]	-31.6%	-0.505 [-0.586, -0.424]	-30.8%
Total opportunity for sexual selection ($w_1w_2w_3$)	$\boldsymbol{I}$	**0.345 [0.154, 0.537]**	**100%**	**1.641 [0.747, 2.535]**	**100%**

Partitioning the selection differential

Our results for the selection differential (*s*) and the standardized selection differential (*s'*) mirrored our findings from the partitioning of the opportunity for selection. While our estimates of *s* and *s*' failed to achieve statistical significance at the $\alpha = 0.05$ level (Table 2), the breakdown of the total selection differential provides some insight into how selection might affect the sexes differently in Gulf pipefish.

In females, *s* and *s'* on female standard length was generated by the first episode of selection, mating success. This premating phase of sexual selection contributed to a positive covariance between female size and fitness and was 155.1% the magnitude of the total selection differential (Table 2). Even though the selection differential was non-significant at $\alpha = 0.05$ in this case, our observation that mated females were significantly larger than non-mated females (see above) suggests that this pattern is real. Both postmating phases of selection opposed premating selection, so the total selection differential was smaller than the selection differential caused by mating success alone (Table 2). The selection differential arising from embryo survivorship in females was significantly negative (Table 2), bolstering the notion that premating selection opposed postmating selection in female Gulf pipefish in our experiment. This result was consistent with the observation that females with greater mating success, which tended to be larger than females with lower mating success, experienced lower per capita offspring survivorship (Figure 1).

In males, the decomposition of the selection differentials showed that larger males experienced greater reproductive success (Table 2). This result was significant for

the standardized selection (*s*') differential on male standard length and nearly significant for the absolute selection differential (*s*; Table 2). However, the source of selection on males is different from that on females. In males, there was no evidence for a relationship between body size and embryo survivorship. Rather, most of the selection differential arose from the first two episodes of selection, only the second of which was statistically significant (Table 2).

Table 2 Decomposition of selection differentials for males and females. Here we show the contributions of mating success, number of eggs transferred per mate, and embryos survivorship to the total selection differential for standard length. We show both the absolute selection differential (*s*), in cm, and the standardized selection differential (*s*'), in units of phenotypic standard deviations, for each sex. Decompositions were performed separately for each experimental tank, and we show means across tanks as well as 95% confidence intervals (in brackets).

Selection Episode	Male *s* [95% CI]	%	Male *s*' [95% CI]	%	Female *s* [95% CI]	%	Female *s*' [95% CI]	%
Premating selection (mating success)	0.034 [-0.012, 0.079]	41.9	0.087 [-0.046, 0.220]	53.5	0.140 [-0.015, 0.295]	143.5	0.209 [-0.011, 0.429]	155.1
Postmating selection (eggs per mate)	0.037 [-0.001, 0.075]	46.0	0.051 [0.006, 0.096]	31.4	-0.026 [-0.093, 0.041]	-26.9	-0.044 [-0.174, 0.086]	-32.8
Postmating selection (embryo survivorship)	0.010 [-0.044, 0.064]	12.1	0.024 [-0.053, 0.102]	15.0	-0.016 [-0.029, -0.004]	-16.6	-0.030 [-0.056, -0.005]	-22.4
Total selection differential	0.080 [-0.002, 0.163]	100	0.162 [0.004, 0.321]	100	0.097 [-0.046, 0.241]	100	0.135 [-0.072, 0.341]	100

Discussion

Our most interesting results concerned the operation of selection in females, the strongly sexually selected sex in the sex-role-reversed Gulf pipefish. Our data showed that almost all of the selection in females was attributable to premating rather than postmating selection in our experimental populations. We found this result to be

surprising, because several studies have shown that postmating selection can occur in pipefish (Partridge et al. 2009; Silva et al. 2009; Paczolt and Jones 2010; Mobley et al. 2011). However, until the present study, no experiments in pipefish (and few in any species) had quantified both pre- and postmating selection episodes in the same individuals as part of a single experiment. Our comprehensive approach revealed tradeoffs between pre- and postmating phases of selection in female Gulf pipefish, which were not apparent in previous studies of pipefish. In particular, females with greater mating success exhibited significantly lower offspring survivorship compared to singly mated females. Thus, under the environmental conditions of our experiment, females that were favored by precopulatory sexual selection had lower fitness in postmating phases of selection.

Our results also clearly show that selection was operating differently on males and females in our experimental breeding aggregations. In males, the limiting sex for reproduction in Gulf pipefish populations, we found that both the opportunity for selection and the selection differential arose mainly from variation in mating success and number of eggs received per mating, whereas for females almost all selection arose from variation in mating success. The variance in fitness in males arising from differential mating success could be a sign of sexual selection on males if females chose not to mate with some males. Differences among males in number of eggs received, however, could be either sexual selection, if females chose to allocate eggs strategically (Silva et al. 2009), or natural selection, if males with larger brood pouches simply accommodated larger numbers of eggs (Berglund et al. 1986*b*). Regardless, the total opportunity for

selection on males (0.345) was about a fifth the magnitude of the opportunity for selection on females (1.641), suggesting that sexual selection is more important in female than in male Gulf pipefish.

Characterization of the Gulf pipefish mating system

The results of the parentage analysis in our study parallel the findings of previous studies describing the mating system of Gulf pipefish. The vast majority of the males in the experiment mated and each of them received eggs from a single female, a result which mirrors those from microsatellite-based studies of natural populations of Gulf pipefish (Jones and Avise 2001; Jones et al. 2001). Our results also show that males, the choosier sex, tended to mate with larger females, which is the same pattern found in *S. typhle* (Berglund and Rosenqvist 1990) and another polyandrous pipefish, *Nerophis ophidion* (Berglund et al 1986*a*). This choosiness results in many females failing to mate, because females have the potential to produce many more eggs than males can fit into their brood pouches (Berglund et al. 1989; Scobell et al. 2009). In addition, females continuously mature eggs in *Syngnathus* pipefish, so mature females are almost always immediately capable of mating upon collection from the wild (Begovac and Wallace 1987). Thus, the failure of some females to mate in our experiment is not likely a consequence of female infertility.

One interesting limitation on reproduction in sex-role-reversed pipefishes is that females compete for access to mates but also bear a substantial cost of producing large eggs (Fitzpatrick et al. 1995). This energetic cost implies that successful females may experience constraints associated with the rate at which they are able to produce eggs

(Berglund et al 1989; Scobell et al. 2009; Braga Goncalves et al. 2011). Indeed, work on *S. scovelli*, *S. typhle* and *N. ophidion* shows that females have a potential reproductive rate that is about two to three times higher than that of males (Berglund et al. 1989; Scobell et al. 2009). Thus, even the most successful females never achieve levels of mating success equal to successful males in the most polygynous species with conventional sex roles, in which some males can sire offspring with dozens of females during a breeding season (Le Boeuf 1974; Fabiani et al. 2004). Our results provide two lines of evidence that energetic constraints associated with egg production may play a role in female reproductive success in Gulf pipefish. First, the females that mated multiply transferred fewer eggs per mate on average when compared with the singly mated females (Figure 1), but had more total eggs transferred. Second, the eggs deposited by multiply mated females experienced lower average survivorship across all of their matings compared to the eggs deposited by singly mated females, suggesting lower overall survivorship rather than declining survivorship in multiply mated females (Figure 2).

That female mating success trades off with number of eggs transferred per mating and embryo survivorship could be explained by a number of possible processes. The simplest explanation might be that multiply mated females are running out of eggs and transferring some eggs before they are fully ripe, resulting in reduced embryo survivorship. This explanation is not consistent with our results, because we saw no difference in between a female's first and second brood. Our sample size for this comparison was small (n=9), however, as our study was not originally designed to

explore this aspect of female reproductive success. Another possibility is that females exhibit a resource tradeoff between investment in precopulatory displays and egg quality. Females may also strategically allocate their egg investment, in turns of number or quality, among males in a process analogous to strategic sperm allocation in species with strongly sexually selected males (Dewsbury 1982; Wedell et al. 2002). Recent work showing that female *S. typhle* females can alter the chemical composition of their eggs in response to male size supports this interpretation (Braga Goncalves et al. 2010). However, the reduced embryo survivorship we observed for multiply mating females could be partially explained by a male-mediated strategy, if males can sense their brood size and invest fewer resources in smaller broods. The patterns of brood reduction we observed in this experiment are probably not a consequence of genetic incompatibility (Zeh and Zeh 1996), as we would expect these incompatible mating events to be equally likely for all females regardless of whether they mated once or multiple times. Regardless of the underlying mechanism, and despite these apparent costs of multiple mating, females with multiple mates had significantly higher fitness than singly mated females, because each additional mate added much more fitness than the females lost due to the reduced number of eggs transferred per mate or decrease in egg survivorship.

The relationship between premating and postmating phases of selection

Our results showed that the three episodes of selection in our experiment were not equally important in determining the total strength of selection. Our most interesting result is that postmating selection, arising from number of eggs transferred and embryo survivorship, made a small contribution to the total opportunity for selection in females.

This result is especially surprising in light of recent studies that suggest that postmating selection is potentially important in Gulf pipefish and in the related broad-nosed pipefish, *S. typhle*. In Gulf pipefish, a previous laboratory experiment showed that males prefer to mate with larger females, males accept more eggs per mating from larger females, and eggs from larger females are more likely to result in viable offspring (Paczolt and Jones 2010). However, that experiment differed from the present study in that it employed a no-choice mating design, in which some males were paired with small females for mating, which isolated the effects of postmating phenomena without the potentially obscuring effects of precopulatory sexual selection. Indeed such an approach is frequently used in studies of postcopulatory sexual selection. In the present experiment, we allowed both premating choice and postmating processes to occur in the same breeding aggregation. Consequently, males may have been able to mate with preferred females in almost every case, reducing the extent to which postmating mechanisms would become necessary in the current study. This conclusion is bolstered by the observation that rates of embryo survivorship were much higher in the present study than in the previous no-choice experiment of postmating selection (93 vs. 71%, respectively).

Our experimental design may have increased the likelihood of detecting a greater role of premating as opposed to postmating selection because of two important factors. First, we equalized the sex ratio and used only females that displayed their secondary sexual characteristics and were thus deemed ready to mate at the start of the experiment. As a consequence, males had a choice of many attractive females, and the fact that

several females mated with three males suggests that the females in each tank collectively had far more eggs than the males had brooding space to accommodate. Second, our studies were conducted at a much higher breeding density than probably occurs in nature. As a result, each male had the opportunity to assess every female in the tank and mate with the best of the eight available females. We do not know how many females a male can sample in the field, but males likely incur greater costs from sampling additional mates in their natural habitats, which include predators and lower population density, than they did in our experiment. Overall, we conclude that postmating selection was not very important in the ecological setting simulated by our experimental tanks. Furthermore, because males receive eggs from only one female per pregnancy, postmating processes in Gulf pipefish do not include sperm or egg competition within a pregnancy. This aspect of the mating system also likely reduces the importance of postmating processes in Gulf pipefish compared to other species of syngnathids. Our study spanned only the length of a single male pregnancy, whereas male pipefish have repeated pregnancies during a prolonged mating season in nature. Tradeoffs between male pregnancies could add another layer of complexity to postmating selection in Gulf pipefish. Thus, the extent to which postmating selection is generally important in Gulf pipefish remains an open issue, and a key question is how often males resort to mating with less attractive mates in natural populations due to demographic or ecological constraints.

The present study and the few others of its kind (Droge-Young et al. 2012; Collet et al. 2012; Pischedda and Rice 2012) represent the next step in understanding the

relative roles of precopulatory sexual selection and its interaction with postmating processes, including phenomena that fall into the realms of postcopulatory sexual selection, sexual conflict and natural selection. While no generalities have yet emerged due to the low number of studies to date, our results and those of Collet *et al.* (2012) lead us to predict that the relative importance of pre- versus postmating selection will be context dependant. We hope that researchers will adopt a standard approach to quantify sexual selection and measure total selection that facilitates easy comparison among studies. Arnold and Wade's (1984*a*, 1984*b*) decomposition of the opportunity for selection and the selection differential provides an ideal framework. Detailed quantitative study of mating patterns and reproductive success promises to resolve the relative contributions of premating and postmating processes, as well as interactions between them, to total sexual selection.

3. THE EFFECTS OF SYNTHETIC ESTROGEN EXPOSURE ON PREMATING AND POSTMATING SELECTION IN SEX-ROLE-REVERSED GULF PIPEFISH*

Introduction

Endocrine disrupting chemicals can mimic natural hormones and alter bodily processes regulated by the endocrine system, causing detrimental effects on reproduction and hormone production (Orlando and Guillette, 2007). The earliest studies on the impacts of endocrine disruptors focused primarily on the negative physiological effects of these compounds on the reproductive systems of exposed animals, particularly in aquatic organisms such as frogs and fish (Allen et al. 1999; Iguchi et al. 2001). These studies documented partial feminization or complete sex reversal in exposed males and termination of egg production in females (Islinger et al. 2003; Länge et al. 2001; Xu et al. 2008). One endocrine disruptor that has received recent attention is a synthetic estrogen found in hormonal contraceptives called 17α-ethinylestradiol, also known as EE2. This contaminant is resistant to degradation in the body and ultimately ends up in the aquatic environment by passing through domestic wastewater treatment facilities and being released as a biologically active molecule in the effluent where it can accumulate to levels that cause deleterious effects on exposed organisms (Koplin et al. 2002). For instance, EE2 has been detected in U.S. rivers at levels as high as 820 ng/L, European

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locations at 35 ng/L, and at 42 ng/L in Canadian sewage treatment effluent (Kolpin et al. 2002; Pojana et al. 2007; Ternes et al. 1999). These high contamination levels raise questions regarding the types of effects that varying levels of EE2 exposure could have on populations of economically or ecologically important species that occupy such sites during all or part of their lifecycles (Segner et al. 2003; Soares et al. 2009).

Lower ranges of EE2 have been detected in surface waters near sewage treatment plants, generally ranging from below 0.5 to 5 ng/L in locations worldwide (Allen et al. 1999; Ternes et al. 1999; Johnson et al. 2000). Chronic exposure to lower concentrations of EE2 has been shown to cause entire populations of fathead minnow (*Pimephales promelas*) populations to stop reproducing after a single generation of 5-6 ng/L exposure (Kidd et al. 2007). Relatively low levels of EE2, ranging from 0.5 - 5.0 ng/L, have been shown to have drastic effects on gene expression levels, reproductive development, and behavior in exposed populations (Ferreira et al. 2009; Larsen et al. 2008; Nash et al. 2004; Soares et al. 2009). Even if endocrine disruptors have no obvious morphological or physiological effects, changes in either gene expression levels or behavior may hinder the organism's reproductive success.

Exposure to endocrine disruptors, such as EE2, can alter an organism's mating behaviors at several different levels of biological organization. For example, endocrine disruptors can improperly induce or eliminate secondary sex traits, decrease sexual behaviors such as courtship displays or male territory aggression, disrupt communication between the sexes, or simply sterilize the exposed animals by interfering with the normal development of reproductive structures (Coe et al. 2008; Munakata and Kobayashi,

2009; Orlando and Guillette, 2007; Sarristo et al. 2009*a*, 2010). In medaka fish (*Oryzias latipes*), for instance, EE2 has been shown to disrupt mating rituals, such as dancing and following with their mates; however, normal behaviors such as resting and swimming were not affected (Oshima et al. 2003). Endocrine disruptors have been shown to alter courtship displays and aggression in several other species of fish including goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), three-spined sticklebacks (*Gasterosteus aculeatus*), salmon (*Oncorhynchus masou)*, and guppies (*Poecilia reticulata*) (Bjerselius et al. 2001; Colman et al. 2009; Bell 2001; Sarria et al. 2011; Kristensen et al. 2005). Several of these studies demonstrated drastic changes in male behaviors, such as males completely failing to court females or no longer defending their territories, at EE2 concentrations as low as 0.5 – 3 ng/L (Bjerselius et al. 2001; Colman et al. 2009; Oshima et al. 2003). Sarristo et al. found that EE2 exposure affects courtship, aggression, and parental care in male sand gobies, *Pomatoschistus minutus* (Saaristo et al. 2009*a*, 2010). Additionally, male sand gobies exposed to EE2 experienced difficulty defending their nests, causing females to prefer non-exposed males when given the choice (Sarristo et al. 2009*a*). This study demonstrates the strong impact EE2 can have on an exposed male's mating success by disrupting the mating system and decreasing the importance of secondary sexual characteristics in males.

We chose to study the effects of EE2 on sexual selection in the sex-role-reversed Gulf pipefish, *Syngnathus scovelli*, because this species resides in areas that are likely to be affected by EE2 and has previously been the focus of many behavioral studies. Gulf pipefish are found in seagrass beds along the Gulf of Mexico and Atlantic coastlines of

North America (Dawson, 1985) and depend on the seagrass community for their habitat, food, and protection. This reliance on the seagrass ecosystem ties the organisms to locations near the coast, often in the vicinity of sewage treatment plants and other sources of environmental contamination. There are numerous benefits to using the Gulf pipefish mating system to test the effects of EE2 on the strength of sexual selection. The Gulf pipefish, like the other members of the Family Syngnathidae, exhibits an evolutionarily novel trait in male pregnancy and as a result is sex-role reversed, meaning that females compete for access to mates and males act as the choosy sex during courtship. Females of this species are typically larger than males at sexual maturity, and males prefer larger females (Jones and Avise, 2001). Gulf pipefish are sexually dimorphic with respect to secondary sexual traits as well, with sexually mature females exhibiting iridescent bands on their trunk, a deeply keeled abdomen, and an enlarged, darkened dorsal fin (Dawson, 1985). None of these traits normally occur in males. The Gulf pipefish has a polyandrous mating system, where males typically mate with a single female per pregnancy but successful females transfer eggs to multiple mates (Jones and Avise, 1999). Consequently, sexual selection acts more strongly on females (Jones et al. 2001). Pregnant male Gulf pipefish carry eggs in a sealed brood pouch, located on the ventral surface of his body, where the eggs that have been transferred from the female are encased and fertilized, resulting in assured paternity of the offspring (Jones et al. 2001). Unsuccessful eggs, or those that do not develop, persist within the brood pouch until the end of the pregnancy, making it easy to accurately count the number of successful and unsuccessful eggs transferred (Paczolt and Jones, 2010).

Our work builds on several studies that have addressed the effects of endocrine disruptors on various species of pipefish (Ripley and Foran, 2008; Sarria et al. 2011*b*). For example, Sarria et al. (2011*c*) found that the juvenile black striped pipefish, *S. abaster*, when exposed to both tributyltin (TBT) and EE2, altered their swimming patterns and their response to the mosquitofish (*Gambusia affinis*), a potential predator. The Gulf pipefish has recently emerged as a useful model for testing the effects of endocrine disruptors. For instance, EE2 levels of 1 ng/L and 100 ng/L have been shown to affect male morphology, gene expression levels, and mating dynamics in the Gulf pipefish (Udea et al. 2005; Partridge et al. 2010). As a result of EE2 exposure, male pipefish experience feminization by developing a deeper abdomen and iridescent bars, traits typically only seen in females. Female Gulf pipefish prefer non-exposed over exposed males, and exposed males were less likely to become impregnated when chosen as a mate (Partridge et al. 2010). These results from binary mate choice assays indicate that mating dynamics in Gulf pipefish are affected by EE2 exposure. Sex-role reversed pipefish have also been shown to have high levels of natural estrogen in brooding males, a reversal of the normal pattern of estrogen in male teleosts, suggesting exposure to a synthetic estrogen, EE2, might affect selection acting on male pregnancy (Mayer et al. 1993). The next question, which we address here, concerns how this disruption in mating preferences alters the mating system and the strength of selection in pipefish breeding aggregations. We set out to determine the effects of low levels of EE2 exposure on both pre- and post-mating episodes of selection in artificial breeding colonies of Gulf pipefish in a laboratory setting. To accomplish this goal, we measured mating success,

reproductive success, and embryo survivorship within small breeding aggregations of Gulf pipefish, which were either exposed or not exposed to low concentrations of EE2 and used these data to measure the effects of EE2 on the intensity of selection and other reproductive attributes of this sex-role-reversed species.

Methods

Gulf pipefish were collected from coastal seagrass beds near Aransas Pass, Texas (27°53'39.07"N, 97° 7'51.69"W) from July through October, 2010. Sexually mature males and females were separated by sex, acclimated to 26ppt salinity tanks, and group housed in a flow-through system at Texas A&M University. All males were collected pregnant to confirm a history of successful reproduction and were allowed to give birth in the laboratory. Males were used in the experiment within a month of their collection to ensure a recent pregnancy. The EE2 concentrations at the collecting site are currently unknown. However, the location was chosen because of its long distance from any outflows from sewage treatment plants.

To determine the levels of EE2 exposure for our experiment we performed a pilot study at EE2 concentrations of 2 ng/L and 5 ng/L with a total of eight males and eight females per treatment along with a parallel control set of fish. We placed males into the experiment while still pregnant with their broods from the field and monitored their abilities to carry their broods to term and to become pregnant with subsequent broods. While control males gave birth and mated soon thereafter as anticipated, several males exposed to 5 ng/L of EE2 experienced difficulties giving birth and had dead offspring in their pouches after the first few days of exposure. None of the males exposed to 5 ng/L

of EE2 had a second pregnancy in the lab and they appeared to resorb their brood pouches, apparently terminating their reproductive activities. Females, in contrast, continued to show courtship displays, including dancing and twitching, similar to the control females. Thus, we concluded from this pilot study that a concentration of 5 ng/L of EE2 would result in complete reproductive failure for Gulf pipefish, apparently mediated by a loss of reproductive ability for males and chose 2 ng/L as our EE2 concentration to allow for more typical pipefish mating and offspring development.

Over the course of the experiment, we conducted seven experimental replicates and seven control replicates. Each replicate began with eight non-pregnant, adult males and eight adult females in a 100 liter tank. For the experimental tanks, we used a 2ng/L EE2 concentration, whereas the control tanks were EE2 free. The 17α-ethinylestradiol powder, of 98% purity, was obtained from Sigma (# 028K1411, MW 296.4, CAS 57-63-6) and dissolved in ethanol. Tanks treated with EE2 were initially dosed on the first day to obtain a concentration of 2 ng/L, and 10% water changes were conducted daily to maintain a constant level of 2 ng/L exposure as established by Partridge et al. (2010). Control tanks were treated with the same volume of ethanol (100µl) without 17α-ethinylestradiol. We used 100 liter tanks that were optimized for length (92 cm x 30 cm), rather than height (38 cm), and were taller than previous tanks (28 cm) used for mating trials in Gulf pipefish (Paczolt and Jones, 2010) to allow a large amount of surface area for fish matings while minimizing the amount of EE2 wastewater.

On the first day of the experiment, fish were marked with three visible implant fluorescent elastomer tags (VIFE; Northwest Marine Technology, Inc.) for identification

using the protocol of Woods and Martin-Smith (2004). After anesthetization with diluted clove oil, each fish was marked on its tail with at least one blue and one yellow band to minimize color differences among marks. The marking procedure produced no mortalities, and we found no evidence for preferences for a single marking pattern during the experiment across both treatments (C: $F_{7,54} = 0.911$, $P = 0.5062$; E: $F_{7,55} = 1.369$, $P = 0.2401$). Fish were then randomly placed into treatments and were not significantly different in size across the two treatments (t-tests, males: n= 109, p = 0.6781; females: n = 111, n = 0.0904).

For three days before the establishment of mixed-sex breeding aggregations in the 100 L tanks, we housed males and females separately from one another and exposed them to the desired level of EE2 (i.e., 2 ng/L for experimental animals and 0 ng/L for control animals). On the fourth day of the experiment, sexes were combined in 100 liter tanks and the EE2 treatments were maintained throughout the rest of the experiment. Each replicate with eight males and eight females per tank was given two weeks during which mating took place. Males were checked daily for pregnancies and sacrificed using MS222 on day eight of their pregnancy. Eggs were dissected out of the male's pouch to determine the number of developing offspring and failed eggs. The proportion of normally developing embryos in each brood is used as a measure of embryo survivorship. Four offspring were removed from the top and bottom of the pouch and preserved in ethanol to use for assigning maternity. All non-pregnant males and females were sacrificed on day 18 of the experiment. Dorsal fins from the adult fish were preserved in ethanol for DNA extractions using the Genomic DNA Purification Kit from

Gentra systems (Qiagen). DNA was extracted from the embryos using a Chelex/Proteinase K (20mg/ml) extraction method.

We conducted a microsatellite-based parentage analysis to determine maternity of the broods using three highly variable microsatellites (*micro25.10, micro25.22,* and *micro22.3*) previously developed by for *S. scovelli* (Jones and Avise 1997*a*). Paternity was already known because the offspring were dissected out of the male's brood pouch, where fertilization occurs and males are assured paternity (Jones and Avise, 2001). Microsatellites were amplified for all adults and eight offspring per male with the exception of the eggs from males with zero offspring survivorship, and PCR products were sent for fragment analysis at the Cornell Life Sciences Core Laboratories Center. Microsatellite fragment sizes were measured by an Applied BioSystems 3730xl DNA Analyzer and analyzed using Peak Scanner software. For each male's brood, a maximum of four alleles were represented, two of which matched the paternal genotype and two that represented the maternal genotype. Within each replicate, one of the eight females from the appropriate replicate was unambiguously matched with each set of embryos by exclusion. Maternity was easily assigned for all male broods, except for two males in the control tanks and one in the EE2 whose embryos failed to develop and thus were not amenable to microsatellite analysis. These three failed broods were excluded from further analysis.

Fish were photographed on the first, eighth, and fourteenth days of the experiment to measure standard body length and depth using ImageJ (NIH). Standard body length was measured from the snout of the fish to the end of the caudal peduncle

and standard depth was measured from the anterior end of the dorsal fin to the base of the fish's ventral surface. We calculated the covariance between standard length and relative fitness to measure the absolute selection differential, *s* (reported in cm), and standardized selection differential, *s*' (reported in units of phenotypic standard deviations; Lande and Arnold, 1983). Absolute and standardized selection differentials were calculated across three episodes of sexual selection, broken down into one pre-mating episode pertaining to mating success and two post-mating episodes, including eggs transferred per mate and embryo survivorship (Arnold and Wade, 1984*a*). These latter two episodes could be considered either sexual or natural selection, depending on the mechanisms involved. We also decomposed the variance in relative fitness, also known as the opportunity for selection (*I*), into components arising from these three episodes of selection (Arnold and Wade 1984*a*, 1984*b*). We calculated *s*, *s*', and *I* for each tank individually and reported the means and standard errors for each episode across replicates. Relative fitness for each episode of selection was calculated separately for each replicate tank by dividing each measure of absolute fitness (i.e., number of mates, number of eggs transferred, or number of surviving offspring) by the corresponding mean absolute fitness across all the individuals of the same sex in the replicate. A more detailed analysis of the control tanks, including a more extensive discussion of the interpretation of the various phases of selection, has been published elsewhere (Rose et al. 2013). All other statistical tests were conducted using JMP 9.0.

Results

Effects of EE2 on mating success

The presence of low levels of EE2 did not have an effect on the number of males that mated successfully. Each treatment saw a similar number of males become pregnant: 91% of males mated in the control tanks with only five of the 56 males failing to become pregnant, and 89% of the males mated in the EE2 treatment, where only six of 56 males remained unmated (Figure 3). Parentage analysis of eight eggs per male, four from each end of the brood pouch, confirmed that all males mated with a single female, regardless of treatment. Using an ANCOVA with tank as a random effect, we found no effect of treatment on male size. However, despite a small sample size of unmated males, we did see a significant difference in size of mated and unmated males across the entire experiment (treatment: $F_{1,109:}$ 0.005, $p = 0.945$; mating category: $F_{1,109:}$ 4.06, $p = 0.047$; treatment*mating category: $F_{1,109:}$ 0.074, $p = 0.787$). Mated males averaged 8.73 cm (SE: 0.15) in length, while unmated males were on average 8.28 cm (SE: 0.25) in length.

The number of mated females was slightly higher in the EE2 treatment than in the control, with 61% of females mating successfully (34 out of 56) in the EE2 tanks as compared to 53% (29 out of 55) in the control (Figure 3). The control had more multiply mated females (n=14) than the EE2 replicates (n=11), whereas the EE2 treatment had more singly mated females (n=23) than the control replicates (n=15). Using an ANCOVA with replicate as a random effect, we found no significant effect of treatment and whether or not the female mated on female body length (ANCOVA: treatment: $F_{1,110}$

= 1.43, p = 0.254; female mating category: $F_{1,110} = 3.225$, p = 0.074; treatment*female mating category $F_{1,110} = 0.448$, p = 0.505).

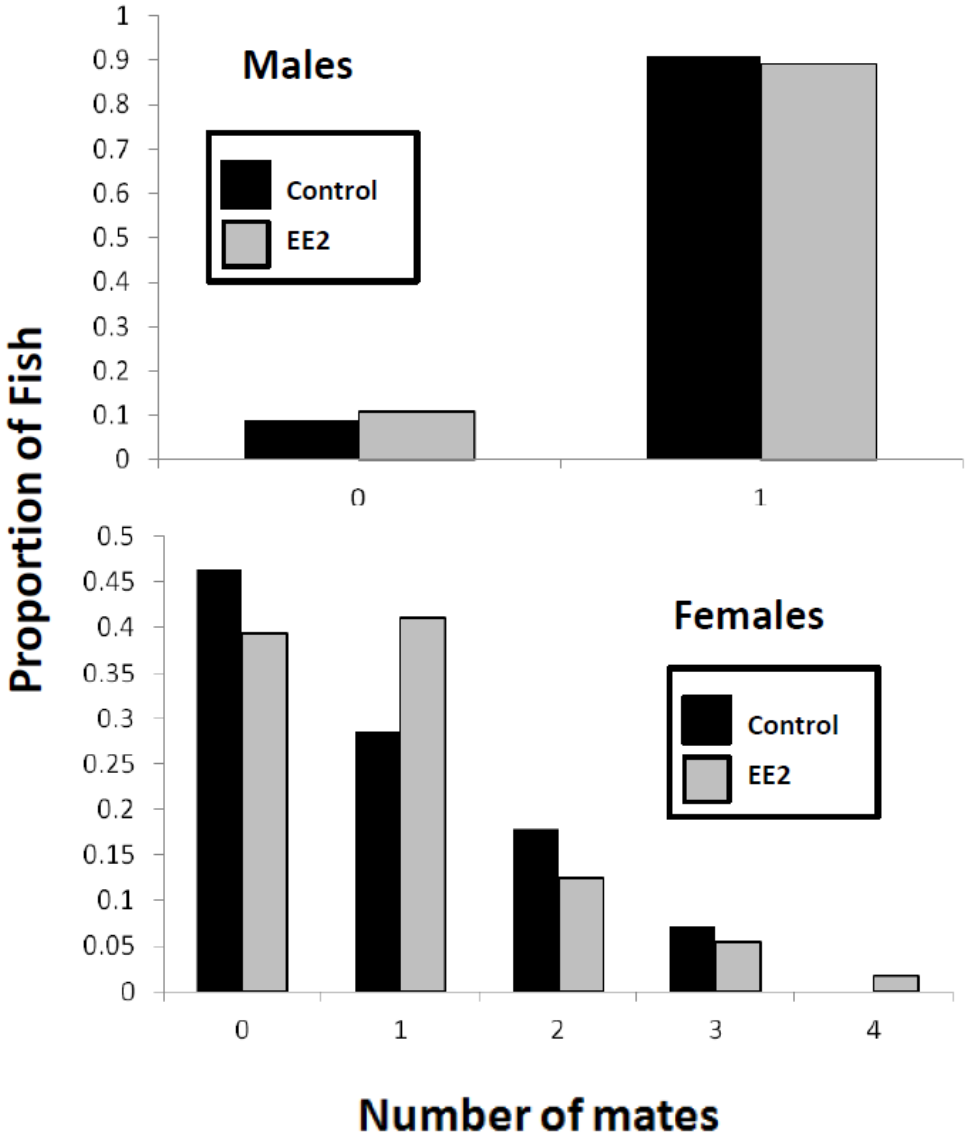

Figure 3 Histograms displaying mating success for male and females across both treatments with black bars for the control and gray bars for the EE2 treatment. The y-axis represents the frequencies and the x-axis represents the number of mates.

Effects of EE2 on reproductive success

To examine female reproductive success, we compared the number of eggs females transferred throughout the whole experiment as a function of treatment and whether the female was unmated, singly mated or multiply mated. We also used female body length as a covariate, included replicate as a random effect, and retained only the significant interactions in the final model (ANCOVA: treatment: $F_{1,11} = 2.17$, $p = 0.164$; female mating category: $F_{2,110} = 246.5$, $p < 0.0001$; female body length: $F_{1,110} = 3.61$, $p = 0.06$; treatment*females mating category: $F_{2,110} = 4.48$, $p = 0.014$). As expected, multiply mated females transferred a greater number of eggs to males over the course of the experiment simply because they had gained access to more total brood pouch space (female mating category: $F_{2,110} = 246.5$, $p < 0.0001$). On average across the treatments, multiply mated females transferred a total of 69 eggs compared to 32 eggs transferred per singly mated female. We also found a significant interaction between treatment and female mating category (treatment*females mating category: $F_{2,110} = 4.48$, $p = 0.014$). This interaction was driven by the multiply mating females in the EE2 experiment transferring a significantly larger number of eggs than any other category of female, including multiply mating females in the control tanks (Figure 4a). Thus, we did not see a difference between treatments in terms of the numbers of eggs transferred by singly mated females (Tukey post-hoc: n = 38, $p = 0.91$), but we did observe that multiply mated females in the EE2 treatment transferred 15 more total eggs on average compared to multiply mated control females (Tukey post-hoc: n = 25, $p = 0.05$).

We compared reproductive success on a per-brood basis across treatments and female mating categories, including singly or multiply mated. We used an ANCOVA to examine the effects of treatment and female mating category on the number of surviving offspring with the total number of eggs initially transferred as a covariate. We included replicate as a random effect and reported only the significant interactions. In the control, females mating with multiple males experienced lower survivorship per embryo relative to singly mated females, whereas in the EE2 treatment no such pattern was evident (ANCOVA: treatment: $F_{1,96} = 0.029$, $p = 0.87$; female mating category: $F_{1,96} = 0.774$, $p = 0.38$; total eggs transferred: $F_{1,96} = 1488$, $p < 0.0001$; treatment*females mating category: $F_{1,96} = 7.66$, $p = 0.007$). Thus, in control tanks, females appear to experience a tradeoff between number of eggs transferred and embryo survivorship, but this tradeoff disappears in the EE2 treatment, despite the fact that multiply mating EE2 females actually transferred a greater number of eggs than multiply mating control females (Figure 4a,b). This pattern is also evident from an embryo survivorship standpoint (Figure 4b): an ANOVA shows a significant difference in embryo survivorship across the experiment ($F_{3,95} = 3.59$, $P = 0.017$).This pattern is a result of a significant difference between embryo survivorship for males mated with singly versus multiply mated females in control tanks (Tukey post-hoc: n = 47, $p = 0.033$), but not between males mated with singly and multiply mated females in EE2 tanks (Tukey post-hoc: n = 50, $p = 0.446$).

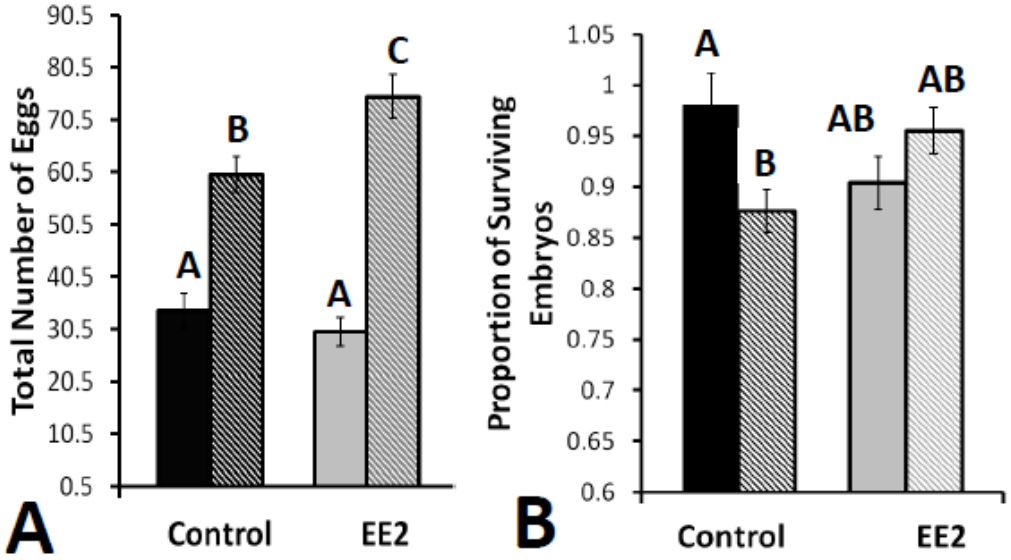

Figure 4 Number of eggs transferred and proportion of surviving embryos for females in the EE2 and control treatments. The graph on the left (a) shows the number of eggs females transferred to their mates over the entire experiment. Solid bars represent singly mated females and striped bars represent multiply mated females. The graph on the right (b) shows the proportion of surviving embryos for singly mated (solid bars) and multiply mated (striped bars) males in the control and EE2 treatments. Within each figure, bars with shared letters are not significantly different from one another (Tukey's post-hoc test). Error bars represent one standard error from the mean.

Effects of EE2 on selection

The decomposition of selection differentials is presented in Table 3. For both treatments, the largest contribution to the total selection on body size in females was from the first episode of selection, mating success. Selection on body size resulting from mating success in the EE2 females was significantly positive at $\alpha = 0.05$, and we observed a similar, non-significant trend in control females. The selection differentials also provide evidence of a tradeoff between pre- and post-mating episodes of selection in control females, a result that was also apparent (and statistically significant) from our

analysis of the relationship between multiple mating and embryo survivorship (see above). For control females, both episodes of post-mating selection, resulting from eggs per mate and offspring survivorship, are negative and oppose the strong positive selection on body length from mating success (Table 3). Interestingly, we saw no evidence for this tradeoff in the EE2 tanks, where selection differentials for pre-mating sexual selection as well as for both post-mating episodes of selection were all positive, favoring larger females (Table 3), but these results also must be interpreted with caution as the 95% confidence intervals for the selection differentials for post-mating episodes overlapped zero. Regardless, our results do clearly show that sexual selection on females was not dramatically reduced as a result of EE2 exposure in Gulf pipefish.

Patterns of selection in males differed considerably from those in females. For instance, no single episode of selection made up the vast majority of the male selection differentials (Table 3). For both control and EE2 males, we see more of a balance between the contributions of the first two episodes. The second episode of selection measures reproductive success per pregnancy and suggests that larger males are receiving a greater number of eggs. This episode is statistically significant in control and EE2 treatments for the standardized selection (*s*') differential on male standard length and is probably best described as fecundity selection, because larger males, which typically have larger brood pouches, receive more eggs per mating (control: $n = 49$, $r = -0.38$, $p = 0.007$; EE2: $n = 50$, $r = 0.29$, $p = 0.044$).

Table 3 Selection differentials broken down into three episodes of selection for the control and EE2 treatments. We calculated the absolute selection differential (s), in cm, and the standardized selection differential (s'), in units of phenotypic standard deviations, for each tank separately and calculated means and 95% confidence intervals (bracketed values below the mean) across tanks. Episodes of selection include mating success, number of eggs transferred per mate, and embryo survivorship.

Control:

Selection Episode	Male *s* [95% CI]	%	Male *s*' [95% CI]	%	Female *s* [95% CI]	%	Female *s*' [95% CI]	%
Pre-mating selection (mating success)	0.034 [-0.012, 0.079]	41.9	0.087 [-0.046, 0.220]	53.5	0.140 [-0.015, 0.295]	143.5	0.209 [-0.011, 0.429]	155.1
Post-mating selection (eggs per mate)	0.037 [-0.001, 0.075]	46.0	0.051 [0.006, 0.096]	31.4	-0.026 [-0.093, 0.041]	-26.9	-0.044 [-0.174, 0.086]	-32.8
Post-mating selection (embryo survivorship)	0.010 [-0.044, 0.064]	12.1	0.024 [-0.053, 0.102]	15.0	-0.016 [-0.029, -0.004]	-16.6	-0.030 [-0.056, -0.005]	-22.4
Total selection differential	0.080 [-0.002, 0.163]	100	0.162 [0.004, 0.321]	100	0.097 [-0.046, 0.241]	100	0.135 [-0.072, 0.341]	100

EE2:

Selection Episode	Male *s* [95% CI]	%	Male *s*' [95% CI]	%	Female *s* [95% CI]	%	Female *s*' [95% CI]	%
Pre-mating selection (mating success)	0.037 [-0.006,0.081]	26.8	0.082 [-0.053, 0.216]	38.0	0.120 [0.029, 0.211]	77.4	0.236 [0.037, 0.436]	86.1
Post-mating selection (eggs per mate)	0.100 [-0.016, 0.215]	71.4	0.133 [0.005, 0.261]	62.0	0.029 [-0.022, 0.080]	18.7	0.030 [-0.050, 0.109]	10.8
Post-mating selection (embryo survivorship)	0.002 [-0.015, 0.019]	1.8	0.0 [-0.019, 0.019]	0.0	0.006 [-0.009, 0.021]	3.9	0.008 [-0.014, 0.031]	3.1
Total selection differential	0.139 [0.013, 0.266]	100	0.215 [0.067, 0.363]	100	0.155 [0.037, 0.272]	100	0.274 [0.050, 0.499]	100

We also calculated the total opportunity for selection and found that the decomposed episodes of *I* paralleled the results from our selection differentials (Table 4). The total opportunity for selection, as well as *I* for each of the three individual

episodes, did not differ across the treatments for either sex. However, similar to our total selection differentials, we see that in both the control and EE2 treatments the total opportunity for selection is statistically greater in females than in males as evidenced by non-overlapping 95% confidence intervals (Table 4). When we break down the sources of variation responsible for *I* into episodes, there are very similar patterns across each treatment within the sexes when compared with our decomposed selection differentials. Similar to *s* and *s*', we find that the first episode of selection, resulting from variance in mating success, is responsible for the majority of variance in female fitness and is statistically significant in both the control and EE2 replicates. On the other hand, the second and third episodes of selection only represent 4-4.6% and 0.6-0.9%, respectively, of the total opportunity for selection in females across treatments (Table 4). The total opportunity for selection in males was significantly lower than that in females, and the decomposition in males reveals different contributions of the various episodes of selection. In general, we found similar patterns in control and EE2 treatments for males. In particular, mating success (I_1) and number of eggs per mate (I_2) make the largest contribution to variation in fitness for males. Embryo survivorship (I_3) made a small (i.e., 4-17.5%) but statistically significant contribution in both treatments (Table 4).

Table 4 Decomposition of the opportunity for selection (I) by selection episode. The selection episodes include number of mates, number of eggs transferred per mate, and offspring survivorship during the pregnancy. The decomposition of I follows Arnold and Wade (1984a, b), and the covariance terms are shown for completeness. See Arnold and Wade (1984a, b) for a more complete discussion of the interpretation of the various terms. For our purposes, the most important terms are I1, I2, and I3, which indicate the variance in relative fitness arising from our three episodes of selection. We conducted this partitioning for each tank separately and calculated means across tanks. We also report 95% confidence intervals (shown in brackets) across tanks.

		Control Tanks				EE2 Tanks			
Source of Variance in Fitness	**Symbol**	**Male Value [95% CI]**	**Male %**	**Female Value [95% CI]**	**Female %**	**Male Value [95% CI]**	**Male %**	**Female Value [95% CI]**	**Female %**
Precopulatory sexual selection (mating success, w_1)	I_1	**0.149 [-0.037, 0.335]**	**43.2%**	**1.582 [0.878, 2.287]**	**96.4%**	**0.132 [-0.001, 0.266]**	**34.0%**	**1.204 [0.631, 1.778]**	**75.8%**
Postcopulatory selection arising from number of eggs transferred (eggs per mate, w_2)	I_2	**0.098 [0.049, 0.147]**	**28.4%**	**0.065 [0.022, 0.108]**	**4.0%**	**0.194 [0.058, 0.329]**	**49.8%**	**0.074 [0.028, 0.119]**	**4.6%**
Covariance between w_1 and w_2:									
Unweighted	$COI(1,2)$	0.105 [-0.013, 0.224]	30.5%	0.519 [0.383, 0.655]	31.6%	0.102 [0.001, 0.203]	26.2%	0.471 [0.328, 0.614]	29.6%
Weighted by number of mates	$COI(1,2\|1)$	0.000 [0.000, 0.000]	0%	-0.064 [-0.157, 0.028]	-3.9%	0.000 [0.000, 0.000]	0%	0.073 [-0.045, 0.192]	4.6%
Change in covariance between number of eggs (w_1w_2) and eggs per mate (w_2) caused by precopulatory sexual selection	$COI(12,2\|1)$ - $COI(12,2)$	-0.097 [-0.210, 0.017]	-28.0%	-0.528 [-0.651, -0.404]	-32.2%	-0.094 [-0.187, -0.001]	-24.2%	-0.358 [-0.507, -0.210]	-22.5%
Variance in number of eggs (w_1w_2)	**Subtotal: I_{12}**	**0.256 [0.072, 0.439]**	**74.1%**	**1.574 [0.806, 2.341]**	**95.9%**	**0.334 [0.207, 0.461]**	**85.8%**	**1.464 [0.764, 2.165]**	**92.2%**
Postcopulatory selection, embryo survivorship (embryo success, w_3)	I_3	**0.060 [0.004, 0.117]**	**17.5%**	**0.009 [0.002, 0.016]**	**0.6%**	**0.016 [0.004, 0.028]**	**4.0%**	**0.014 [0.002, 0.026]**	**0.9%**
Covariance between number of eggs (w_1w_2) and embryo success (w_3):									
Unweighted	$COI(12,3)$	0.122 [0.000, 0.244]	35.3%	0.540 [0.406, 0.674]	32.9%	0.150 [0.046, 0.254]	38.7%	0.481 [0.334, 0.627]	30.2%
Weighted by number of eggs	$COI(12,3\|2)$	0.016 [-0.001, 0.033]	4.6%	0.023 [-0.032, 0.077]	1.4%	0.021 [0.000, 0.043]	5.4%	0.055 [-0.009, 0.119]	3.4%
Change in covariance between total fitness ($w_1w_2w_3$) and embryo success (w_3) caused by first two episodes of selection	$COI(123,3\|2)$ - $COI(123,3)$	-0.109 [-0.218, 0.000]	-31.6%	-0.505 [-0.586, -0.424]	-30.8%	-0.132 [-0.230, -0.035]	-34.0%	-0.425 [-0.555, -0.295]	-26.7%
Total opportunity for sexual selection ($w_1w_2w_3$)	I	**0.345 [0.154, 0.537]**	**100%**	**1.641 [0.747, 2.535]**	**100%**	**0.389 [0.228, 0.549]**	**100%**	**1.589 [0.807, 2.371]**	**100%**

Our final metric related to sexual selection was the Bateman gradient, which we calculated for females and compared across treatments (Figure 5). Our results show that the Bateman gradient for females is significantly steeper in the EE2 treatment than it is in the control treatment (ANCOVA with replicate as a random effect*: p*= 0.009), implying that sexual selection may be slightly stronger in the EE2 treatment than in the control treatment.

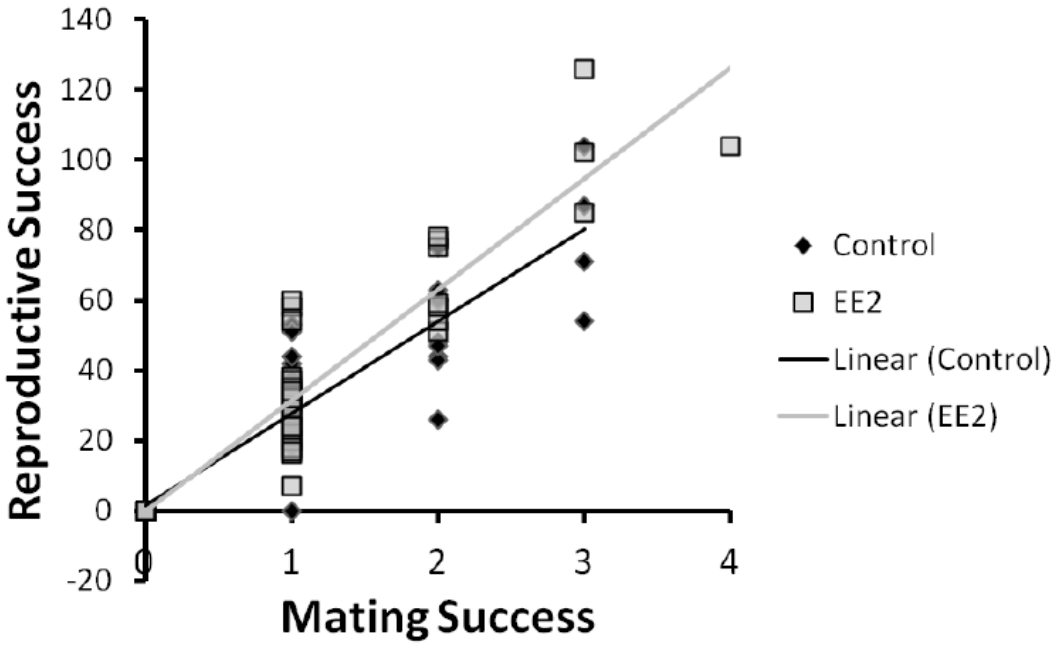

Figure 5 Absolute Bateman gradients for females in the EE2 and control treatments. The black line represents the linear regression of total number of offspring on number of mates for the control treatment, whereas the gray line shows the same linear regression for the EE2 treatment. The Bateman gradient for females in the EE2 treatment is significantly steeper than the Bateman gradient for females in the control replicates (ANCOVA, with replicate as a random effect: p= 0.009).

Discussion

Exposure to EE2 has previously been shown to alter the mating behaviors of Gulf pipefish in binary choice tests, which suggested that EE2 could potentially disrupt the mating system and alter sexual selection. While we did find several changes in the mating system of Gulf pipefish as a result of low levels of EE2 exposure, we did not see a significant change in the strength of selection for either sex. The presence of 2 ng/L of EE2 in our experimental treatments did not hinder the ability of males to become pregnant. However, exposure to higher concentrations of EE2, including 5ng/L in our pilot study and 100 ng/L in previous studies, even for a short period of time, significantly impacted male reproductive potential (Partridge et al. 2010). While EE2 concentrations of 5 ng/L are typically considered to be on the lower end of the range of EE2 levels detected in the natural environment, studies have shown chronic exposure to these relatively lower levels of EE2 can sterilize male fishes (Koplin et al. 2002; Kidd et al. 2007). Thus, high levels of EE2 could certainly result in population collapse due to a complete loss of male brooding in pipefish. However, under the lowest levels of EE2 exposure investigated in the present study, males were able to become pregnant and the nature of sexual selection acting on females remained mostly unchanged relative to control populations.

The most important observation in our study indicated that EE2 exposure affected the reproductive success of females, but the proximate effect appeared to be positive rather than the negative effect we would have predicted *a priori*. Females in the control group that mated multiply transferred fewer eggs per mate than females with

only one mate, indicating that females may have rationed their eggs or become egg limited as they mated with additional males. In the case of EE2-exposed females, however, we found a different pattern: multiply mated females transferred a comparable number of eggs per mate compared to singly mated females. When we look at the total number of eggs produced by females we see that females exposed to EE2 that mated multiply produced many more eggs overall than the multiply mated females in the control group. The multiply mated females did not differ in body length between the treatments, so this difference cannot be attributed simply to larger females having higher fecundity, a trend typically seen in other species of pipefish (Braga Goncalves et al. 2011). The most likely explanation for this observation is that exposure to low levels of EE2 stimulates egg production in female Gulf pipefish thereby increasing their reproductive rates and that females can only realize these enhanced reproductive rates by mating with multiple males. This interpretation is consistent with observations involving fathead minnows, in which short periods of EE2 exposure have been shown to increase eggs production (Jobling et al. 2003). However, chronic low levels of exposure eventually cause decreased egg production in fathead minnows (Jobling et al. 2003).

Several studies have also shown reproductive failure in zebrafish as a result of either long-term exposure to low levels of EE2 or brief exposure to higher levels (Nash et al. 2004; Van den Belt et al. 2003; Xu et al. 2008), raising the possibility that the short-term benefit that we observed in pipefish could be offset by reduced lifetime fitness for EE2-exposed females. Future studies involving a longer timeframe will be necessary to address this question.

The increase in reproductive rates in EE2-exposed females also appeared to have an effect on the tradeoff between pre- and post-mating episodes of selection. In the control group, females that mated multiply were able to transfer more eggs than singly mated females over the entire experiment but in turn had more of their eggs fail to develop. This tradeoff between greater mating success in the pre-mating phase of selection and a decrease in offspring survivorship, a post-mating mechanism, was not present in the EE2 multiply mated females. This observation raises the possibility that EE2-exposed females produce eggs of higher quality as a result of the excess estrogen resource. It is also important to note that while we did not dissect out the ovaries of unmated females, there did not visually appear to be any degradation of functioning ovaries in the females that did not mate.

As noted above, while an exposure of 2 ng/L seems to benefit females in the short-term, EE2 exposure at 5 ng/L, a concentration on the lower side of the range of EE2 detected in the natural environment, results in devastating reproductive impacts on Gulf pipefish populations. Females are largely unaffected, but males are seriously compromised by this level of EE2 contamination. In our pilot study, males exposed to 5 ng/L failed to carry pregnancies to term, were unable to mate, and showed abnormal brood pouch morphology. At higher levels of exposure, the effects are even more dramatic. For instance, male Gulf pipefish exposed to 100ng/L displayed secondary sexual traits that are normally only found in females, including iridescent bands and a deeply keeled abdomen. Furthermore, these males showed a reduced ability to mate even after being removed from the short-term EE2 exposure, with a minimum lag time of four

days until pregnancy (Partridge et al. 2010). Thus, we predict that exposure in natural populations approaching 5 ng/L or higher will reduce the reproductive potential of Gulf pipefish populations and that the negative effects will be mediated almost entirely by the impact of EE2 exposure on males rather than on females. Several studies have documented levels of EE2 ranging from 5 ng/L and below, indicating that both of the concentrations in our study are within the range of possible EE2 contamination occurring in the environment where natural populations of the Gulf pipefish reside.

We did not see a breakdown in selection in the Gulf pipefish's sex-role-reversed mating system in populations exposed to EE2. Instead, we see no significant changes in the opportunity for selection in both male and female Gulf pipefish. As a result of increased female reproductive rates in EE2, we actually see a small but non-significant increase in selection acting on body length in both males and females. In a previous study by Sarristo et al. (2009*b*) documenting a breakdown in sexual selection in sand gobies, a species with strong sexual selection on males, sexual selection was disrupted due to the feminization of males. If the male traits that sand goby females use to choose their mates, such as aggressiveness and courtship displays, are disrupted by EE2 exposure, then sexual selection would be expected to break down. In our sex-role-reversed system, we did not detect a breakdown of selection as a result of male feminization. If anything, selection on females was slightly stronger in the EE2 treatment than in the control (as evidenced by the significantly steeper Bateman gradient for EE2 exposed females), which could be due to the greater number of eggs available and their increased survivorship, possibly causing an increase in female-female

competition. Sand gobies have conventional sex roles with male-male competition for access to females, so it makes sense that feminization of males would reduce their ability to compete for mates. Part of the reason we found a different effect of EE2, compared to the complete collapse of selection acting on male sand gobies, certainly stems from the sex-role-reversed mating system of Gulf pipefish. Increased feminization of females appears to have made them more fecund and more able to compete for mates. However, feminization of male Gulf pipefish did not seem to affect the mating system until the males began to lose their ability to maintain functional brood pouches, a situation that results in a complete cessation of reproduction rather than quantitative changes in the intensity of sexual selection. It is also important to note that in the study conducted by Sarristo et al. (2009*b*) female sand gobies were not exposed to EE2. However, in the present study we exposed both sexes to EE2 to investigate the effects of exposure in a setting that would more closely mimic contamination of the natural environment.

In summary, low levels of EE2 exposure enhanced reproduction in female Gulf pipefish and increased the fitness of multiply mated females during both pre- and post-mating episodes of selection. EE2 exposure did not disrupt pre-mating sexual selection in the Gulf pipefish mating system. If anything sexual selection was slightly stronger in the exposed populations, owing to the increased fecundity of exposed females. Even though this study documented the effects of low levels of EE2 exposure on the Gulf pipefish mating system, it is critical to put the level of exposure in perspective. In our study, EE2 had a positive effect on female egg production at low levels of 2ng/L. However, at EE2 exposure levels of 5 ng/L and higher, male receptivity decreased and

male fitness plummeted to zero. In conclusion, this study has added to our knowledge on the effects of EE2 on pipefish, providing more insight into the effects EE2 has at the population level rather than on the individual level. Now that we have established that successful matings can occur under low levels of EE2 exposure, the next question is how these pollutants affect these fish at various stages of their lifecycle, especially in terms of juvenile recruitment and population viability in EE2 contaminated waters.

4. THE EFFECTS OF SYNTHETIC EXPOSURE ON THE SEXUALLY DIMORPHIC LIVER TRANSCRIPTOME OF THE SEX-ROLE-REVERSED GULF PIPFISH[*]

Introduction

Sex-role-reversed species, in which females tend to evolve elaborate secondary sexual traits and males tend to be choosy, have enjoyed a rich history in the study of the evolution of sex differences and sex roles. In the sexual selection literature, sex-role reversal is usually defined as the situation in which sexual selection acts more strongly on females than on males (Vincent et al. 1992), and it is normally associated with substantial parental investment by males (Trivers 1972), which reduces the male potential reproductive rate below that of the female (Clutton-Brock and Parker 1992; Kvarnemo and Ahnesjö 1996). Sex-role-reversed species have provided unique opportunities to study sexual selection, because they have allowed novel tests of hypotheses related to mating competition and have challenged ideas related to the meaning of maleness and femaleness. While sex-role-reversed mating systems are quite rare in most taxonomic groups, sex-role reversal has nevertheless been documented in fishes, amphibians, birds, and insects (reviewed in Eens and Pinxten, 2000). Work over the last several decades has elucidated many of the ultimate mechanisms responsible for the evolution of sex-role reversal, such as effects of parental investment on the operational sex ratio, the environmental potential for polygamy, and the Bateman

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[*] Reprinted with permission from The effects of synthetic estrogen exposure on the sexually dimorphic liver transcriptome of the sex-role-reversed Gulf pipefish by Emily Rose, Sarah P. Flanagan, Adam G. Jones. 2015. *PLoS One* 10:10. Copyright 2015 by Rose et al. DOI:10.1371/journal.pone.0139401

gradient (Emlen and Oring 1977; Kvarnemo and Ahnesjö 1996; Jones et al. 2001). However, many of the proximate effects of sex-role reversal are not well understood, even though we know that the evolution of sex-role reversal is accompanied by potentially dramatic changes in morphology, behavior, and reproductive physiology.

The phenotypic manifestations of sex-role reversal include traits such as showy ornaments and increased aggression in females, as well as marked mating preferences in males (Berglund and Rosenqvist 1993). In addition, sex-role-reversed species also often have adaptations associated with male parental investment (Bonduriansky 2001). Many of these obvious external features might be expected to be accompanied by underlying metabolic or physiological changes (Eens and Pinxten 2000; Scobell and MacKenzie 2011). Because the traits associated with sex-role reversal tend to be sexually dimorphic, their expression should be encoded by genetic pathways that are connected to the sex-determination hierarchy. While little is known about the mechanisms underlying the genetic basis of sexual dimorphism, previous studies have shown that genes on the sex chromosomes and genes regulated by the hypothalamic-pituitary-gonadal axis, such as growth hormone (GH), are often involved in regulating size dimorphisms (Mei and Gui 2015). In vertebrates, such connections involve sex hormones, so most of the studies aimed at understanding proximate mechanisms regulating sex-role-reversed morphology and behavior have focused on the endocrinology of sex-role reversal, with a particular emphasis on birds (Eens and Pinxten 2000; Adkins-Regan 2012).

The endocrinology of sex-role-reversed species has perhaps generated more questions than answers. One general pattern is that female maturation and cycles of

gamete production are controlled by estrogens, whereas male primary sexual traits are controlled by androgens, in much the same way as reproduction is controlled in species with conventional sex roles (Eens and Pinxten 2000; Scobell and MacKenzie 2011). However, efforts to pin down the proximate causes of female-specific morphology and behavior in sex-role-reversed taxa have revealed a complex picture that appears to vary among taxa. For instance, in most species of sex-role-reversed birds, males have higher testosterone levels than females (Staub and De Beer 1997), but there is some indication that testosterone levels nevertheless modulate female aggression, perhaps because certain regions of the female brain are more sensitive to androgens compared to the male brain (Voigt and Goymann 2007). On the other hand, in some sex-role-reversed birds, such as barred buttonquails and moorhens, males and females exhibit similar plasma levels of testosterone (Eens et al. 2000; Muck and Goymann 2011), so general patterns regarding testosterone's role have yet to emerge. In addition, hormones other than testosterone have been implicated in sex-role reversal in birds. For example, progesterone modulates female aggression in the black coucal (Goymann et al. 2008), and males have higher prolactin levels than females in spotted sandpipers (Oring et al. 1986) and Wilson's phalarope (Oring et al. 1998), a reversal of the levels normally observed in species with conventional sex roles. Few studies have been conducted on the endocrinology of non-avian sex-role-reversed species, but what little work has been done indicates a complex picture in these other taxa as well. For instance, in sex-role-reversed populations of the peacock blenny (a marine fish), estradiol and prostaglandin F2α are involved in female courtship displays and mating behavior (Gonçalves et al.

2014). In short, the endocrinology of sex-role reversal appears to be complex and remains poorly understood.

One noticeable void in the study of proximate mechanisms of sex-role reversal exists in the realm of transcriptome-level responses of sexually dimorphic tissues to hormonal manipulation. Here we begin to fill this gap by studying the transcriptome of the Gulf pipefish (*Syngnathus scovelli*) liver exposed to the potent endocrine disruptor 17α-ethinylestradiol (EE2). We chose to study the Gulf pipefish because it has become an excellent model for the study of many aspects sex-role reversal, and it is an emerging model in the realm of sex-role-reversed endocrinology (Scobell and MacKenzie 2011). Several important features of the liver motivated us to focus on this organ, rather than other obvious choices such as the gonads or male brood pouch, for this initial analysis of transcriptome-level sexual dimorphism and response to an environmental estrogen. First, unlike testes and ovaries, the liver is present in both males and females, facilitating a meaningful comparison of transcriptomes between the sexes. Second, livers in general have been shown to be sexually dimorphic (Roy and Chatterjee 1983; Hirao et al. 2011; Zheng et al. 2013), and in fish the liver in particular plays a major role in reproduction, as many important egg proteins are produced in the liver and transported to the eggs in females. Third, several important liver genes, such as *vitellogenin* and *zona pellucida* isoforms, are known to be estrogen dependent (Sumpter and Jobling 1995) and thus provide clear expectations regarding changes in expression levels in response to elevated levels of estrogenic compounds.

Studies regarding the effects of environmental estrogens in Gulf pipefish and its close relatives provide context for the present study. In particular, we know that external female morphology is estrogen-dependent, because exposure of male Gulf pipefish to 17α-ethinylestradiol (EE2) results in the development of female-like secondary sexual traits (Ueda et al., 2005; Partridge et al. 2010). In addition, expression of *vitellogenin* has been shown to be induced by estradiol or EE2 in four pipefish species and a seahorse, *Syngnathus acus*, *S. scovelli*, *S. abaster*, *Hippocampus guttulatus* (also known as *H. ramulosus*) and *Nerophis lumbriciformis* (Covens et al. 1987; Ueda et al. 2005; Sárria et al. 2013), indicating that this gene is estrogen-regulated in syngnathid fishes. Sufficiently high levels of EE2 (above about 5 ng/L) prevent males from developing a functional brood pouch and consequently cause complete reproductive failure (Partridge et al. 2010; Rose et al. 2013). This observation is consistent with other data from unpublished dissertation work, which indicates that the male brood pouch is androgen-dependent (reviewed in Scobell and MacKenzie 2011). However, thus far no evidence suggests that EE2-exposed males adopt female-typical courtship behavior (Sárria et al. 2013), so the proximate mechanisms of sex-role reversal in pipefish seem to mirror those of avian taxa in terms of complexity. As a first step toward understanding transcriptome-level responses to hormone manipulation in a sex-role-reversed pipefish, we used RNA-sequencing (RNA-seq) to characterize the liver transcriptomes of female, pregnant male, and non-pregnant male Gulf pipefish exposed to the potent endocrine disruptor EE2. Our first goal was to examine patterns of sexual dimorphism in liver gene expression in non-exposed control animals to identify genes displaying sexual

dimorphism or responsiveness to male pregnancy status. We hypothesized that genes involved directly in egg production would show much higher levels of expression in females than in males, but we also recognized the possibility that constraints imposed by male pregnancy could result in the production of additional compounds in the livers of either males or females. For example, if females package additional proteins in their eggs to function during post-copulatory processes, potentially occurring within the male's brood pouch, or if males provision their offspring, then some important proteins involved in these processes could conceivably be produced in the livers of either sex.

Our second goal was to quantify gene expression changes in the liver in response to estrogen exposure in females, pregnant males, and non-pregnant males. We hypothesized that the general pattern of expression changes would be toward feminization of male livers. However, if multiple endocrine signaling pathways have been altered to different degrees by the evolution of sex-role reversal, then we might expect an intermediate pattern, in which some genes are very strongly affected by EE2 while others are relatively resistant even though they are nevertheless sexually dimorphic in their expression patterns. We further assessed whether our observed gene expression patterns are sex-role reversed by comparing our results to those from the zebrafish (*Danio rerio*), a model organism with conventional sex roles.

Methods

Experimental design and sequencing

Gulf pipefish were collected from Redfish Bay near Aransas Pass, Texas (N 27 53 39.07, W 97 7 51.69) on July 8th, 2013 under the Texas Parks and Wildlife permit

number SPR-0808-307. This study was approved by the Institutional Animal Care and Use Committee at Texas A&M University (Animal Use Protocol # 2013-0020, Reference #001898) and fish were killed using an overdose of MS-222. We collected only pregnant males and females with well-developed iridescent bands, ensuring that all individuals were sexually mature. Males were allowed to give birth before being used in the experiment. Fish were dipped in freshwater for ten minutes to remove any external parasites and then acclimated to 26-ppt salinity tanks at Texas A&M University. The 17α-ethinylestradiol powder, of 98% purity, was acquired from Sigma (#SLBF2546V) and dissolved in 200-proof ethanol from Sigma (#SHBD6226V). All tanks held 7 liters of water and were initially dosed with 50 µl of either a 7ng/10µl stock EE2 ethanol solution to have tank concentrations of 5ng/L EE2 or 50 µl of ethanol without EE2 for the controls. The chosen concentration of 5 ng/L EE2 was selected because it is within the range of EE2 found in contaminated bodies of water in nature and also because long-term exposure to this concentration has been shown to cause entire breeding populations of some fish species to collapse (Nash et al., 2004; Kidd et al., 2007; Kolpin et al., 2002). Ten percent water changes were completed daily to ensure that EE2 concentrations remained at a constant 5ng/L as established by Partridge et al (2010).

To generate each pregnant male, we placed one non-pregnant male and one female together in a clean, EE2-free saltwater tank until the male became pregnant. Each pair was also assigned a non-pregnant male, size matched with the pregnant male but not paired with a female. On the second day of the male's pregnancy, all three fish were photographed, measured, and randomly assigned to treatments of either experimental

tanks of 5ng/L EE2 concentrations or the EE2-free control tanks on the second day of pregnancy. The three fish were held in 7L tanks with glass dividers between the fish that allowed for water exchange but otherwise kept the fish isolated from one another. After seven days of exposure, that is, on the ninth day of the male's pregnancy, fish were sacrificed using MS-222 and the liver was dissected. Dissections were done with the fish soaked in RNAlater under a microscope with tissue immediately frozen on dry ice and stored in the -80 ºC freezer. The control and 5ng/L EE2 treatments were replicated 5 times resulting in a total of 30 fish, including 5 females, 5 pregnant males, and 5 non-pregnant males from each treatment (i.e., EE2 and control).

RNA was isolated from the dissected livers using a TRIzol® Reagent (Life Technologies, Carlsbad, CA) extraction method modified from Leung and Dowling (2005). Total RNA was sent to the Michigan State University RTSF Genomics Core where libraries were prepared using the TruSeq mRNA Library Prep Kit v2. Libraries were tested for quality control using Caliper GX and qPCR methods. All 30 individuals were barcoded, allowing for each individual's sequence data to be recovered. The 30 libraries were sequenced using two lanes of an Illumina HiSeq 2500 Rapid Run flow cell v1. Base calling was done by Illumina Real Time Analysis (RTA) v1.17.21.3 and output of RTA was demultiplexed and converted to FastQ with Illumina Bcl2fastq v1.8.4.

Transcriptome assembly

A total of 805 million 150 bp paired end reads were obtained from the MSU Genomics core and were then trimmed with Trimmomatic (Bolger et al. 2014) using the following settings: HEADCROP:12, LEADING:10, TRAILING:10,

SLIDINGWINDOW: 4:15, MINLEN: 50. All of the paired, trimmed files were next run through the program FLASH to identify and collapse overlapping reads before starting the assembly (Magoc and Salzberg 2011). All of these prepared reads from the 30 individuals were assembled into a single transcriptome with Trinity using version trinityrnaseq_r20140717 and the default parameters (Grabherr et al. 2011). The Trinity assembly consisted of a total of 174,578 Trinity transcripts and 130,728 Trinity 'genes', referred to as contigs throughout the paper. The Trinity assembly had an N50 of 1866, an average contig size of 973 nucleotides, and a total of 169,973,948 assembled bases. From the Trinity assembly, the contig with the longest open reading frame (ORF) per 'gene' was retained in the final transcriptome. Rsem was used to map the paired, trimmed reads from all of the individuals to the retained contigs, hereafter referred to as the "liver transcriptome" (Li and Dewey, 2011). The contig counts (FPKM) from Rsem were then analyzed using EBSeq to identify differentially expressed contigs across the treatments and categories within the treatments (Leng et al., 2013). Finally, the contigs reported by EBSeq to be differentially expressed were blasted against the non-redundant protein database at NCBI. We also used Blast2Go to ascertain Gene Ontology (GO) functions (Conesa et al., 2005). To assess the completeness of our transcriptome, we used the program CEGMAv2.5, Core Eukaryotic Gene Mapping Approach, and recovered 244 complete sequences and 247 partial gene sequences from the CEGMA database containing 248 core eukaryotic genes, using a default e-value of 10 (Parra et al. 2007). From these CEGMA results, we can conclude that our liver transcriptome has a very high level of completeness, because the majority of the conserved core eukaryotic

genes are represented. This CEGMA analysis was employed only to assess the completeness of our transcriptome assembly; all other analyses used the full set of 130,728 Trinity 'genes'.

Analysis

After assessing differential expression in all pairwise treatment comparisons, we identified genes that had at least a 2-fold expression difference between control females and control males. These genes were considered to be sexually dimorphic, and their expression patterns were analyzed in all groups, including EE2-exposed fish, using heat maps. Heat maps were generated in R (R Core Team 2015) using the package gplots (Warnes et al. 2015). We also utilized a principal components analysis (PCA) using prcomp in R (R Core Team) to compare gene expression patterns across the treatments. For this analysis, we removed all contigs that had less than the mean number of reads mapped to them (3,090 reads) across the 30 fish from the two treatments.

To address whether the expression patterns we found showed signs of sex-role reversal, we compared our results to those from a similar study in zebrafish (Zheng et al. 2013), a species with conventional sex roles. The zebrafish study exposed males and females to water containing 5 μg/L 17β-estradiol (i.e., a 1000-fold higher concentration than we used in our study) for 48 hours and investigated transcriptome expression in the liver. We were interested in whether female pipefish have similar gene expression patterns to zebrafish females, and whether males exposed to estrogens respond similarly in species with conventional sex roles (zebrafish) and reversed sex roles (pipefish). Venn diagrams were created to compare the number of genes with female-biased expression

patterns in control females and the number of up-regulated or down-regulated genes in estrogen-exposed males. To carry out the comparisons between species, gene names were generalized by removing subunit isoform types and by grouping some gene families.

Results

Sexually dimorphic gene expression patterns

The RNA-seq results identified a total of 482 Trinity gene transcripts that were significantly differentially expressed in the control female livers compared to the livers of control pregnant and non-pregnant males, with a false-discovery-rate corrected $p \leq 0.05$. Of these 482 differentially expressed transcripts, 67% were up-regulated in females (325 contigs) and the remaining 33% showed higher expression levels in males (157 contigs) (S1). The gene ontology analyses in Blast2GO identified two biological processes, cellular process and metabolic process, as being the most common in the control female liver transcripts. These two processes accounted for 22 and 19 percent, respectively, of the biological processes represented in the annotated sequences (S1).

Of the 325 up-regulated transcripts in females, a total of 21 genes showed an over-expression of 20 fold or higher compared to males (Table 5). These over-expressed genes included several known female-specific genes, such as *vitellogenin b* and *c*, *choriogenin h*, and *zona pellucida sperm-binding protein 4-like*, which have all been shown to be involved in egg development and maturation (Tata 1976; Wassarman 1988; Murata et al. 1997). In addition to the female-specific genes, *estrogen receptor alpha* (*esr1*) was also highly up-regulated in control females. Several of the genes with the

largest differences in expression levels, such as *extracellular serine threonine kinase fam 20c-like* (*Fam20c*), were labeled as "cellular response to estrogen stimulus" genes in the Blast2GO analysis. Many of these strongly sexually dimorphic genes have also been shown to have female-biased hepatic expression profiles, particularly in response to estrogen exposure (Hoffmann et al. 2006). Examples include *3-hydroxy-3-methylglutaryl-coenzyme a reductase* (*hmgcra*), *methylsterol monooxygenase* 1(*msmo1*), and *lanosterol 14-alpha demethylase-like* (*CYP51A1*), in addition to the aforementioned female-specific genes. Of the 157 genes that were up-regulated in males, only one transcript, *histidine triad nucleotide-binding protein 3-like* (*hint3,* S1), exhibited a greater than 20-fold difference in expression levels between males and females.

A comparison of the transcripts between the sexes by absolute number of reads showed that 46 of the top 50 expressed genes were shared between the two sexes (Table 6). Females had four genes within their 50 highest expressed transcripts that were not included within the males' top 50, including transcripts for a translationally controlled tumor protein and three egg proteins: *vitellogenin b*, *zona pellucida sperm-binding protein 4-like*, and *chorion protein*. The males also had four genes that were found solely in the list of 50 top expressed genes for males but not females. These genes included *pancreatic elastase*, *coagulation factor VIIb precursor*, *fibrinogen alpha chain-like*, and a hypothetical unknown protein gene.

Table 5 Hepatic genes with female biased expression patterns in control fish livers. List of blastx annotation and fold change information for all genes showing female-biased expression patterns with fold changes of 20 or higher in control females relative to control males.

Top Blastx Hit Description	Fold Change
vitellogenin c	1588
c44657_g1_i1	1579
extracellular serine threonine protein kinase fam20c-like	1207
vitellogenin b	1031
brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2-like	792
c48786_g1_i1	692
cathepsin e-like	635
choriogenin h	528
extracellular serine threonine protein kinase fam20c-like	458
extracellular serine threonine protein kinase fam20c-like	456
zona pellucida sperm-binding protein 4-like	239
estrogen receptor alpha	47
c55348_g2_i1	40
3-hydroxy-3-methylglutaryl-coenzyme a reductase	39
c38044_g1_i1	34
methylsterol monooxygenase 1	30
sodium- and chloride-dependent creatine transporter 1-like	30
c43284_g1_i1	29
sodium- and chloride-dependent creatine transporter 1-like isoform x1	23
lanosterol 14-alpha demethylase-like	21
monocarboxylate transporter 13-like	20

Table 6 Top 50 expressed genes in the male and female livers for control fish. The top blastx hit descriptions, contig numbers, and mean number of reads for control males (the first set of 50 genes listed) and control females (the second set of 50 genes listed) are shown. The bold contig IDs represent the four sex-specific genes for each sex.

	Top 50 Expressed Genes	**Contig ID**	**Mean # reads**
1	warm temperature acclimation-related 65 kDa protein	c16137_g1_i1	313030
2	ApoA-I	c41331_g1_i1	161667
3	TPA: hypothetical protein BOS_23215	c20770_g1_i1	232799
4	PREDICTED: histidine-rich glycoprotein-like	c33611_g1_i1	294179
5	complement component C3	c56882_g1_i1	281272
6	hypothetical protein OXYTRI_13058	c27857_g1_i1	260783
7	14 kDa apolipoprotein, partial	c28061_g4_i1	171653
8	transferrin	c48809_g2_i1	112617
9	PREDICTED: betaine--homocysteine S-methyltransferase 1	c54924_g2_i1	129460
10	cytochrome c oxidase subunit I	c59228_g4_i1	144836
11	astacin like metalloprotease	c11350_g1_i1	99607
12	alpha-1-antitrypsin	c45579_g1_i1	103302
13	PREDICTED: myeloid protein 1-like	c48382_g1_i1	98270
14	alpha-1-antitrypsin	c51148_g1_i1	76306
15	chymotrypsinogen 1	c78479_g1_i1	58669
16	PREDICTED: beta-microseminoprotein-like	c52972_g5_i1	71077
17	complement C1q tumor necrosis factor-related protein 3-like	c43914_g2_i1	73256
18	hyaluronan binding protein 2 precursor	c52158_g2_i1	59368
19	PREDICTED: fibrinogen gamma chain	c47758_g1_i1	63278

Table 6 Continued

	Top 50 Expressed Genes	**Contig ID**	**Mean # reads**
20	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	c47414_g1_i1	68241
21	PREDICTED: collagenase 3-like	c58007_g5_i1	65271
22	PREDICTED: type-4 ice-structuring protein-like isoform X2	c33291_g1_i1	52243
23	PREDICTED: bile acid receptor-like isoform X2	c50783_g1_i2	49235
24	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H3-like	c56014_g1_i2	58790
25	trypsin-2 precursor	c6350_g1_i1	49044
26	fatty acid-binding protein	c27863_g1_i1	39381
27	aldolase A	c56993_g6_i1	46269
28	hypothetical protein, partial	c42909_g1_i1	44988
29	PREDICTED: chymotrypsin-like protease CTRL-1	c51234_g1_i1	44034
30	PREDICTED: ceruloplasmin-like	c48515_g2_i6	40423
31	PREDICTED: LOW QUALITY PROTEIN: selenoprotein P	c55131_g1_i1	40219
32	PREDICTED: alpha-2-macroglobulin-like isoform X1	c58355_g3_i1	45394
33	PREDICTED: protein AMBP-like	c52079_g1_i1	46994
34	complement regulatory plasma protein	c51045_g2_i1	40653
35	Pancreatic elastase	**c24626_g1_i1**	32525
36	elongation factor 1 alpha	c43004_g1_i1	36091
37	PREDICTED: prothrombin	c47589_g1_i1	35742
38	PREDICTED: complement C5-like	c46369_g3_i1	34227

Table 6 Continued

	Top 50 Expressed Genes	**Contig ID**	**Mean # reads**
39	PREDICTED: complement factor B-like	c57046_g1_i1	35738
40	coagulation factor VIIb precursor	**c53536_g1_i1**	37228
41	PREDICTED: fibrinogen beta chain	c50315_g1_i1	20373
42	vitellogenin c	c52176_g1_i1	19005
43	PREDICTED: alpha-2-HS-glycoprotein-like	c41324_g1_i1	29738
44	hypothetical protein, partial	**c55432_g7_i1**	36658
45	PREDICTED: alpha-2-HS-glycoprotein-like	c41324_g1_i1	41955
46	plasminogen	c40786_g1_i1	31122
47	hypothetical protein, partial	c12437_g1_i1	24375
48	glyceraldehyde-3-phosphate dehydrogenase	c44637_g1_i1	25244
49	PREDICTED: fibrinogen alpha chain-like	**c55500_g1_i1**	33769
50	PREDICTED: kininogen-1-like	c54367_g1_i1	25964

	Top 50 Expressed Genes	**Contig ID**	**Mean # reads**
1	Ribosomal 40s 18s	c27857_g1_i1	602577
2	vitellogenin b	**c58295_g2_i1**	583908
3	vitellogenin c	c52176_g1_i1	197651
4	TPA: hypothetical protein BOS_23215	c20770_g1_i1	179421
5	PREDICTED: histidine-rich glycoprotein-like	c33611_g1_i1	172732
6	complement component C3	c56882_g1_i1	104763
7	cytochrome c oxidase subunit I	c59228_g4_i1	104364

Table 6 Continued

	Top 50 Expressed Genes	**Contig ID**	**Mean # reads**
8	PREDICTED: betaine--homocysteine S-methyltransferase 1	c54924_g2_i1	86711
9	ApoA-I	c41331_g1_i1	80579
10	14 kDa apolipoprotein, partial	c28061_g4_i1	60526
11	alpha-1-antitrypsin	c45579_g1_i1	59001
12	astacin like metalloprotease	c11350_g1_i1	55898
13	warm temperature acclimation-related 65 kDa protein	c16137_g1_i1	53062
14	PREDICTED: beta-microseminoprotein-like	c52972_g5_i1	46663
15	transferrin	c48809_g2_i1	46224
16	PREDICTED: myeloid protein 1-like	c48382_g1_i1	42133
17	aldolase A	c56993_g6_i1	39493
18	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	c47414_g1_i1	36096
19	elongation factor 1 alpha	c43004_g1_i1	34786
20	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H3-like	c56014_g1_i2	30860
21	trypsin-2 precursor	c6350_g1_i1	29855
22	fatty acid-binding protein	c27863_g1_i1	29508
23	chymotrypsinogen 1	c78479_g1_i1	29312
24	alpha-1-antitrypsin	c51148_g1_i1	28159
25	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H3-like	**c58914_g1_i1**	27712
26	hyaluronan binding protein 2 precursor	c52158_g2_i1	27393

Table 6 Continued

	Top 50 Expressed Genes	**Contig ID**	**Mean # reads**
27	complement C1q tumor necrosis factor-related protein 3-like	c43914_g2_i1	27226
28	PREDICTED: fibrinogen gamma chain	c47758_g1_i1	26773
29	PREDICTED: alpha-2-HS-glycoprotein-like	c41324_g1_i1	25675
30	zona pellucida protein sperm-binding protein 4-like	**c47764_g1_i1**	23924
31	PREDICTED: bile acid receptor-like isoform X2	c50783_g1_i2	23763
32	PREDICTED: collagenase 3-like	c58007_g5_i1	21668
33	PREDICTED: type-4 ice-structuring protein-like isoform X2]	c33291_g1_i1	21626
34	PREDICTED: LOW QUALITY PROTEIN: selenoprotein P	c55131_g1_i1	21530
35	complement regulatory plasma protein	c51045_g2_i1	20297
36	chorion protein	**c41859_g1_i1**	18712
37	PREDICTED: chymotrypsin-like protease CTRL-1	c51234_g1_i1	18248
38	PREDICTED: ceruloplasmin-like	c48515_g2_i6	18197
39	PREDICTED: fibrinogen beta chain	c50315_g1_i1	17399
40	glyceraldehyde-3-phosphate dehydrogenase	c44637_g1_i1	17202
41	PREDICTED: protein AMBP-like	c52079_g1_i1	17066
42	PREDICTED: complement factor B-like	c57046_g1_i1	16969
43	PREDICTED: kininogen-1-like	c54367_g1_i1	16600
44	translationally-controlled tumor protein	**c38295_g1_i1**	15892
45	hypothetical protein, partial	c42909_g1_i1	15834

Table 6 Continued

	Top 50 Expressed Genes	**Contig ID**	**Mean # reads**
46	plasminogen	c40786_g1_i1	15555
47	PREDICTED: prothrombin	c47589_g1_i1	15421
48	PREDICTED: alpha-2-macroglobulin-like isoform X1	c58355_g3_i1	15328
49	PREDICTED: complement C5-like	c46369_g3_i1	14994
50	hypothetical protein, partial	c12437_g1_i1	14889

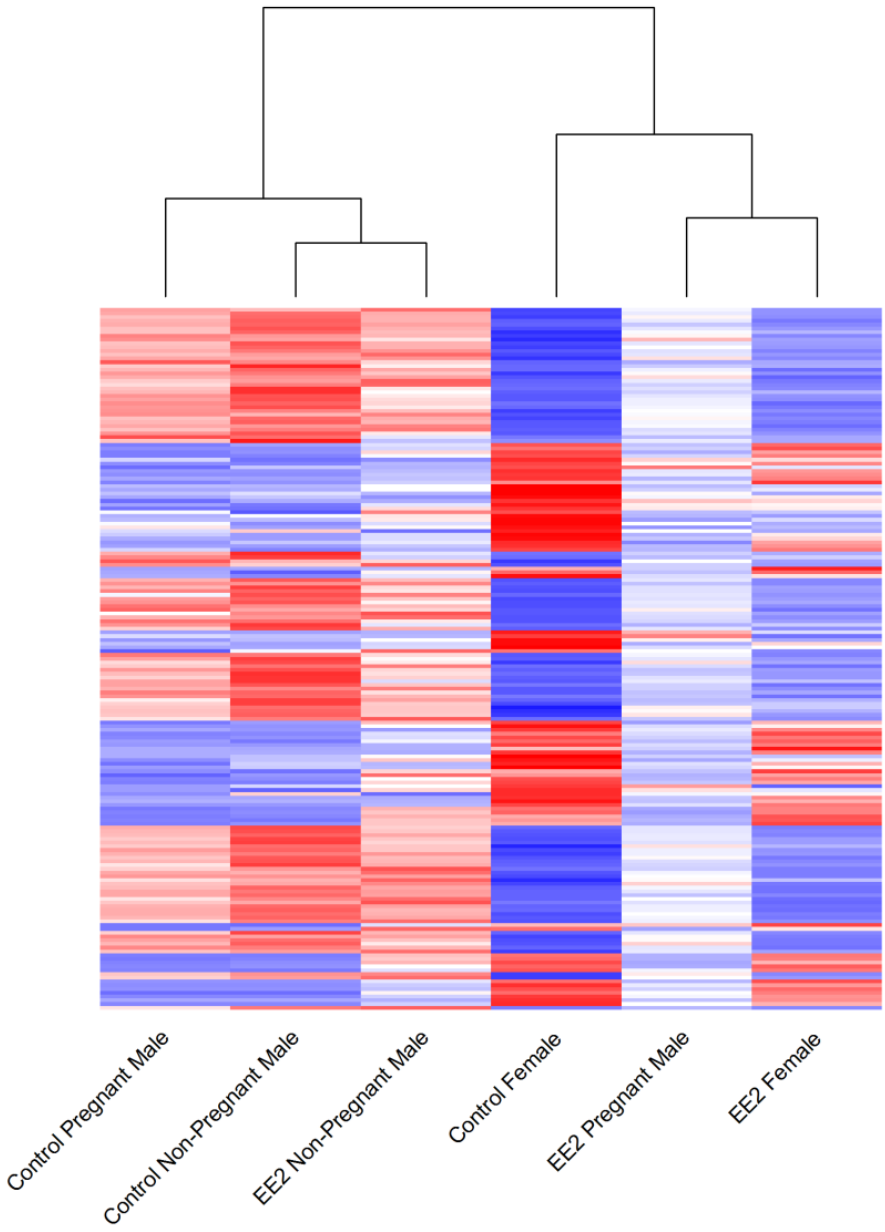

Figure 6 Sexually dimorphic gene expression patterns for all treatments. Heat map showing hierarchical clustering of mean expression values for the six treatments including control females, pregnant males, and non-pregnant males, as well as EE2 exposed females, pregnant males, and non-pregnant males, for all genes which showed sexually dimorphic expression patterns in control fish. The colors of the bars represent either up-regulated (red) or down-regulated (blue) genes. The male treatments cluster together with the exception of the EE2 exposed pregnant males which cluster with the both control and EE2 females.

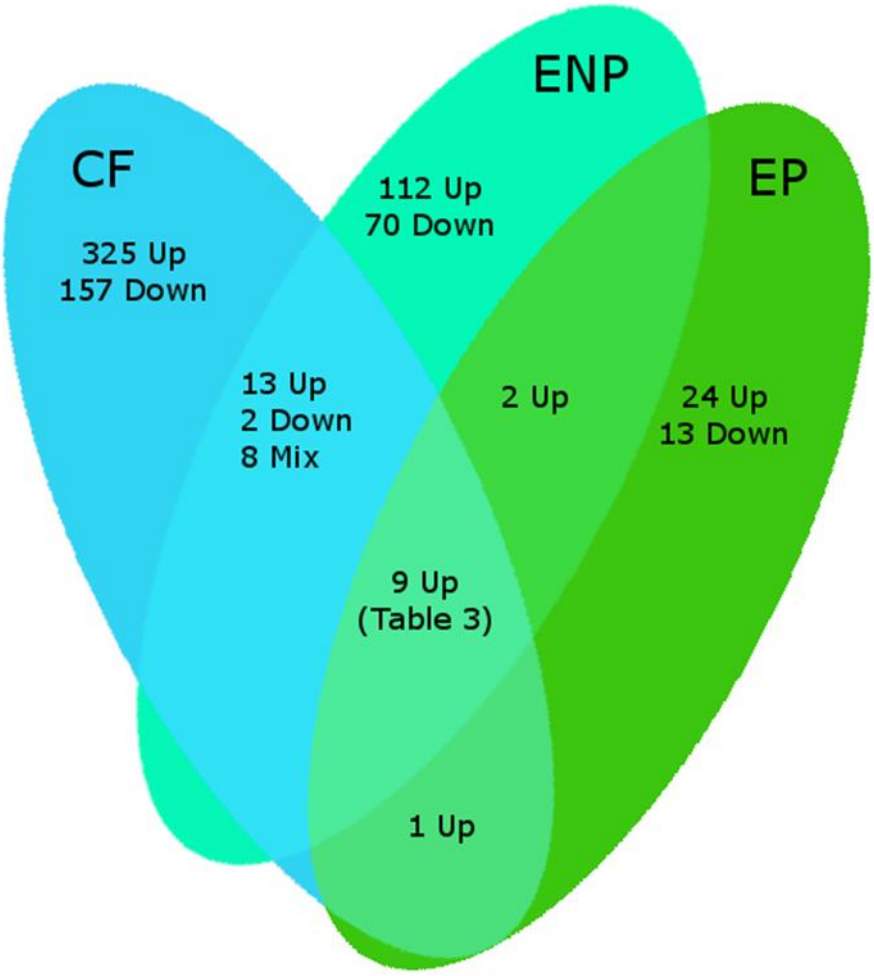

Figure 7 The overlap of female biased and EE2 responsive genes. Venn diagram of control females (CF), EE2 exposed pregnant males (EP), and non-pregnant males (ENP) to show overlap of female biased genes and EE2 responsive genes. Gene expression levels for control females are either over or under expressed compared to the levels of expression in control males, and EE2 exposed male genes were differentially expressed from their control counterparts. A list of genes up-regulated between all three groups can be found in Table 7.

Restricting attention to sexually dimorphic genes, we found that control pregnant males and non-pregnant males had very similar patterns of gene expression to one another that were quite distinct from those of females (Figure 6). This observation supports the hypothesis that both pregnant and non-pregnant males have a male-like gene expression pattern that differs drastically from that of females. A comparison of gene expression between control pregnant and control non-pregnant males showed that 41 genes were differentially expressed between these categories of males. Of these 41 transcripts, two genes (contig c58371_g3_i3 and cornifelin homolog b-like) were upregulated by greater than 20 fold in pregnant males, and one gene (cartilage acidic acid 2) was upregulated greater than 20-fold in non-pregnant males (S2).

Effects of synthetic estrogen exposure

After one week of exposure to 5ng/L EE2, both the pregnant and non-pregnant males' gene expression patterns demonstrated a clear response to estrogen and showed signs of feminization (Figure 6). Non-pregnant males exhibited a greater response to EE2 exposure than did pregnant males, with a total of 182 genes differentially expressed (112 up-regulated and 70 down-regulated) in EE2-exposed non-pregnant males relative to control non-pregnant males (Figure 7, S3). We detected only 37 differentially expressed gene transcripts in exposed pregnant males relative to control pregnant males (24 up-regulated and 13 down-regulated; Figure 7, S4). Two of the genes up-regulated in exposed non-pregnant males, but not in exposed pregnant males, were contig c58371_g3_i3 and *cornifelin homolog b-like*, which also were up-regulated in control pregnant males (S3, S4). A subset of nine genes was up-regulated in control females

(relative to control males), EE2-exposed pregnant males (compared to control pregnant males) and EE2-exposed non-pregnant males (relative to control non-pregnant males). These loci included *cathepsin e-like* (a liver-specific aspartic proteinase), *extracellular serine threonine protein kinase fam20c-like*, and *choriogenin h*, among others (Table 7; Figure 7). Among the subset of nine genes showing both female biased expression and EE2 responsiveness in exposed males, nearly half of these genes were known egg-associated loci, such as *vitellogenin b* and *c*, *choriogenin h* and *l*, and *zona pellucida sperm-binding protein 4-like* (Table 7), which are normally expressed at high levels in females only. A comparison of exposed pregnant males to exposed non-pregnant males revealed one gene that was upregulated greater than 20-fold in pregnant males (contig c59977_g1_i1) and two genes that were upregulated greater than 20-fold in non-pregnant males (*enolase-phosphatase E1-like* and *fibrous sheath CABYR-binding protein-like*; S5).

The livers of EE2-exposed females did not show a pattern of increased feminization relative to livers of control females. Rather, we saw expression patterns respond in the opposite direction. A total of 20 genes were found to be sexually dimorphic in control fish and also differentially expressed between EE2-exposed and control females (Table 8). Eighteen of these 20 transcripts showed a pattern in which the effects of EE2 exposure in female livers were of opposite sign compared to the pattern of sexual dimorphism. For instance, genes that were upregulated in female control fish relative to male controls were usually downregulated in EE2-exposed females relative to control females. However, these effects were relatively small (Table 8), and no

transcripts showed a greater than 20-fold expression difference in females as a result of EE2 exposure (S6).

Table 7 Genes showing both female biased expression in control fish and EE2 responsiveness in exposed males. List of shared, up-regulated genes in control females (when compared with control males) and EE2 exposed pregnant and non-pregnant males (when compared their control counterparts) and their significant fold inductions.

Top Blastx Hit Descriptions	**Control Female**	**EE2 Preg Male**	**EE2 Non-Preg Male**
Upregulated compared to:	Control Males	Control Preg Males	Control Non-Preg Males
vitellogenin c	1587	291	62
choriogenin h	528	67	94
zona pellucida sperm-binding protein 4-like	239	157	83
extracellular serine threonine protein kinase fam20c-like	1207	125	268
brain-specific angiogenesis inhibitor 1-associated protein 2-like	792	66	70
cathepsin e-like	635	141	451
sodium- and chloride-dependent creatine transporter 1-like	30	13	6
protein jagunal homolog 1-b-like	1.2	1.1	1.3
c48786_g1_i1	691	225	139

A principal components analysis (S7) shows a clear separation of control males and females, regardless of female exposure status. The top loadings for PC1, which explains 49-percent of the variation, include *vitellogenin b*, *warm temperature acclimation-related 65 kDa protein*, and *ribosomal protein 40s 18s*, indicating that PC1 captures variation in some of the genes with the largest sex biases in expression. Exposed pregnant males fall between the females and the rest of the males (S7). Overall,

the first three principal components account for 85 percent of the variation, with three of the top genes that represent the highest loadings in PC1 also listing in the top loadings for PC2 and PC3. Restricting attention to the sexually dimorphic genes, we see a general trend for males to become feminized in their gene expression patterns as a result of EE2 exposure (Figure 6). The grouping of EE2-exposed pregnant males with females in Figure 6 and S7 indicates that exposure to synthetic estrogen makes pregnant male livers more female-like than it does to non-pregnant male livers. However, this observation should be interpreted in light of our previous result that EE2 affects the expression patterns of more genes in non-pregnant male livers relative to pregnant male livers.

Table 8 Expression patterns for female EE2 responsive genes. List of blastx annotation and fold change information for all genes showing female-biased expression patterns in control females and EE2-responsive patterns in EE2-exposed females when compared with control females.

Top Blastx Hit Descriptions	Fold Change for Control Females	Fold Change for EE2 Exposed Females
adenylosuccinate synthetase isozyme 1 c-like	1.6	-1.9
cytosolic carboxypeptidase-like protein 5-like isoform x1	1.5	-1.7
40s ribosomal protein s18	2.3	-5.4
myelin protein zero-like protein 3-like	1.2	-1.7
lysosomal acid phosphatase precursor	1.7	-5.0
rna-directed dna polymerase from mobile element jockey-like	1.4	-3.2
warm temperature acclimation protein 65-2	1.7	-7.7
26s protease regulatory subunit 7	-2.3	1.7
angiotensinogen	-3.8	1.7
atpase asna1	-2.9	1.8
camp-regulated phosphoprotein 19	-2.6	1.4
cytochrome c	-4.1	1.8
mitochondrial rho gtpase 2-like	-3.6	1.6
nadh dehydrogenase	-2.4	2.0
peptidyl-prolyl cis-trans isomerase h	-3.3	2.2
proteasome subunit alpha type-3	-2.6	1.7
proteasome subunit beta type-4-like	-3.3	1.7
threonine--trna cytoplasmic	-8.2	2.7
run and fyve domain-containing protein 2-like isoform x1	-1.1	-1.7
solute carrier family facilitated glucose transporter	-4.2	-5.8

Comparison to zebrafish

We found that the majority of the sex-biased genes in zebrafish and pipefish did not overlap, with only nine female-biased genes shared between non-exposed females of the two species (Figure 8a). In the comparison of the down-regulated genes in females, only four were shared between non-exposed zebrafish and pipefish (Figure 8b). Most of the genes that were up-regulated in males of either species in response to EE2 exposure were also female-biased genes (63 of 84 genes in zebrafish males and 11 of 13 genes with 20-fold or higher expression changes in pipefish). The only genes that were female-biased in both species and up-regulated in EE2-exposed males of both species were *vitellogenin 2/vitellogenin b* and *vitellogenin3/vitellogenin c*. When only sexually-dimorphic genes were considered, pipefish males had more genes that were down-regulated than up-regulated in response to EE2 exposure, which was the opposite pattern observed in zebrafish males. Although some of the genes that were down-regulated as a result of EE2 exposure were female-biased (14 of 51 in pipefish and 10 of 57 in zebrafish), the majority were not. In fact, most EE2 responsive, down-regulated genes in zebrafish were male-biased (38 of 57). However, in pipefish, only 11 of 51 down-regulated genes were male-biased.

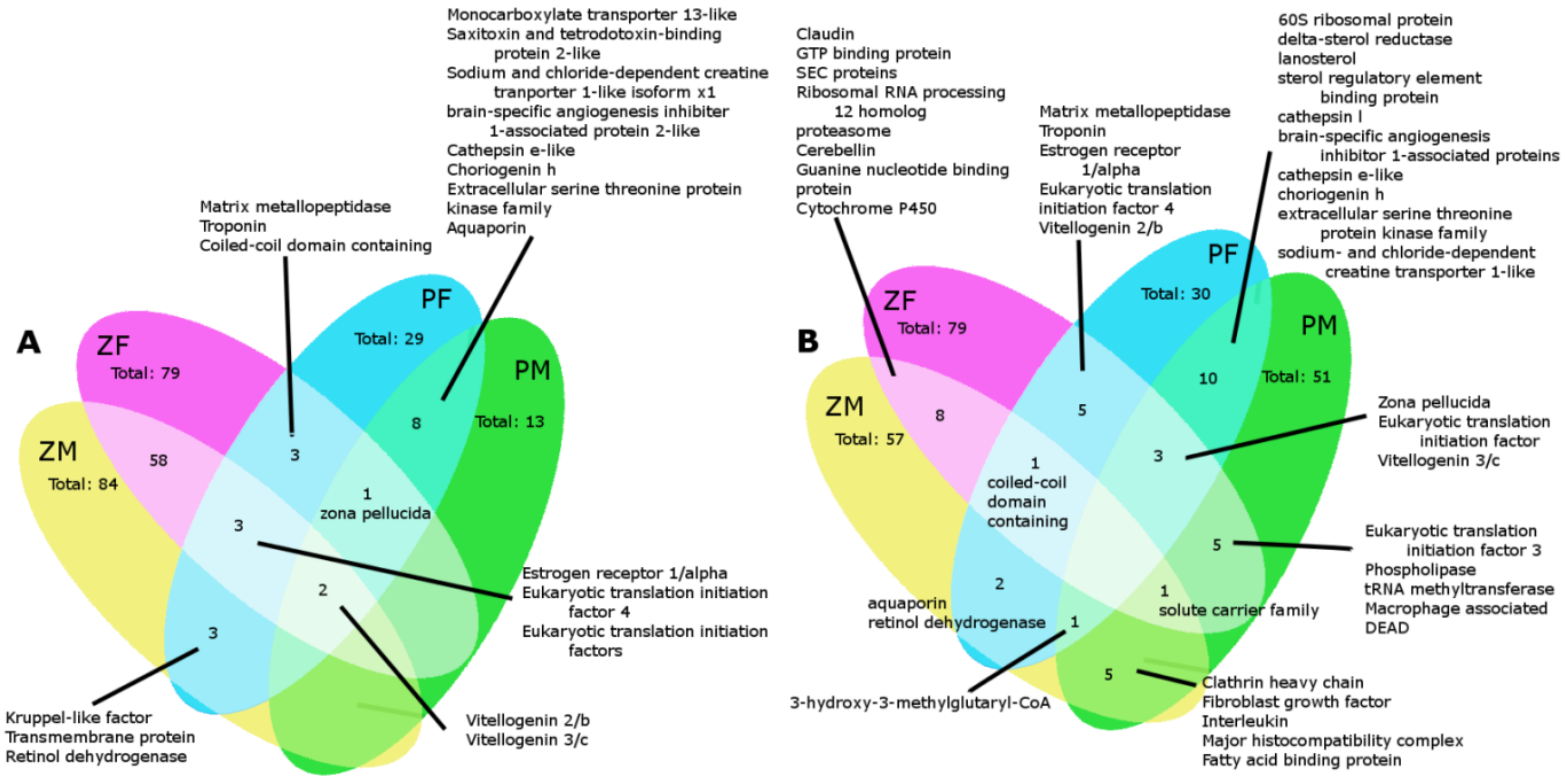

Figure 8 Pipefish and zebrafish comparisons of expression patterns. Venn diagrams of sexually dimorphic and EE2 responsive genes for pipefish and zebrafish showing EE2 up-regulated genes in 3a and EE2 down-regulated genes in 3b. Numbers of female biased genes in control females are presented for control pipefish females in this study (PF) and for control zebrafish from a study by Zheng et al. (ZF). Male EE2 responsive genes are presented for both pregnant and non-pregnant, EE2 exposed pipefish males (PM) and for EE2 exposed males from the Zheng et al. study (ZM).

Discussion

This first study of the effects of a potent endocrine disruptor on gene expression patterns in a sex-role-reversed organism shows that the liver of the Gulf pipefish is markedly sexually dimorphic and responds very strongly to an estrogenic compound. For instance, several female-biased genes respond to EE2 exposure in males in a direction indicative of feminization of the male liver transcriptome. Control fish follow a pattern predicted by the hypothesis that both pregnant and non-pregnant males should differ from females in terms of expression patterns as a consequence of the major role of the liver in producing egg-related proteins. Our results also show that fish exposed to synthetic estrogen show expression patterns consistent with the idea that, even in sex-role-reversed species, female-biased liver genes should be regulated by estrogen. In particular, we see a shift toward more feminine patterns of gene expression in males exposed to EE2. Some aspects of our analysis raise the intriguing possibility that the liver plays a role in not only female reproduction but also male reproduction, as sperm-related transcripts occur in the liver, an observation that leads to the hypothesis that males may also be capable of transporting proteins from the liver to the brood pouch. In addition, as we will explain below, we believe our results leave the door open for sexual selection to act on genes expressed in the liver that ultimately play a role in postcopulatory sexual selection or sexual conflict.

The endocrinology of sex-role reversal

Sex-role-reversed organisms represent an evolutionary challenge from an endocrinology perspective (Eens and Pinxten 2000; Scobell and MacKenzie 2011),

because the females must evolve nuptial ornaments and competitive mating behavior, possibly including enhanced aggression, while simultaneously preserving female reproductive cycles and primary sexual traits. All of these traits related to reproduction and mating competition are potentially sexually dimorphic and hormonally regulated. All sex-role-reversed vertebrates evolved from ancestral taxa with conventional sex roles, and in such taxa androgens (e.g., testosterone or 11-ketotestosterone) are the main hormones involved in almost every aspect of male reproductive competition from the development of primary sexual traits to the modulation of secondary sexual traits and mating behavior (Norris 1997; Ketterson and Van Nolan 1999). Consequently, a male can conceivably become more ornamented or more aggressive during contests for mates by increasing testosterone levels without impairing the male's ability to produce gametes, although testosterone does have other costs (Folstad and Karter 1992). In sex-role-reversed species, in contrast, a female competing for mates cannot increase her competitive ability simply by increasing testosterone levels without suppressing normal female reproduction, so the evolution of sex-role reversal must have involved mechanisms to circumvent this constraint (Eens and Pinxten 2000; Scobell and MacKenzie 2011). The diagnosis of these mechanisms has been difficult and remains a work in progress, so we discuss in turn three aspects of female reproduction, namely reproductive physiology, female ornamentation, and female mating behavior, in light of our results and the broader literature on the endocrinology of sex-role reversal.

Our study is most relevant to female reproductive physiology and indeed represents the first dataset addressing transcriptome-wide effects of hormone

manipulation in any reproduction-related tissue from a sex-role-reversed species. Our results provide the first evidence that the pipefish liver shows genome-wide patterns of dimorphism, with hundreds of genes differentially expressed between males and females. Some transcripts, such as *vitellogenin b* and *vitellogenin c*, showed greater than 1000-fold higher levels of expression in females compared to males. Our EE2 results establish that these expression patterns are largely estrogen-regulated, because males exposed to EE2 expressed genes that were normally only found at high levels in females. Estrogen profiles have been studied in other sex-role-reversed taxa, notably birds, and in general results show that estradiol profiles are similar to non-sex-role-reversed birds (Fivizzani and Oring 1986; Fivizzani et al. 1986; Gratto-Trevor et al. 1990). The emerging pattern is that estrogens control reproductive physiology in females of sex-role-reversed species in much the same way as in females of species with conventional sex roles. Hence, our data are consistent with an emerging pattern that many sexually dimorphic traits are regulated by the hypothalamic-gonadal-pituitary axis (Mei and Gui 2015).

The situation becomes more confusing when we turn attention to ornamentation in sex-role-reversed taxa. In pipefish, female secondary sexual traits occur in males exposed to EE2, suggesting that female ornamentation is controlled by estrogens (Ueda et al. 2005; Partridge et al. 2010; Sárria et al. 2013). However, the pattern is markedly different in sex-role-reversed birds. In Wilson's phalaropes, moorhens and barred buttonquails, injection of females with testosterone increases the brightness of their ornamentation (Johns 1964; Eens et al. 2000; Muck and Goymann 2011). Thus,

secondary sexual characteristics in these species seem to be affected more by androgens than by estrogens. In other sex-role-reversed marine fishes, such as the peacock blenny and the two-spotted goby, neither estrogens nor androgens seem to be responsible for female nuptial coloration, suggesting an altogether different mechanism could be at work (Skold et al. 2008; Gonçalves et al. 2014). In short, sex-role-reversed females seem to have evolved many disparate mechanisms to modulate nuptial coloration, and pipefish may provide a particularly tractable model because EE2 exposure alone is sufficient to produce female-specific secondary sexual traits in males.

Perhaps the most interesting and demanding area of research in the realm of sex-role-reversed endocrinology concerns the modulation of female behaviors involved in mating competition, and researchers have thus far only scratched the surface of this difficult topic. Almost no work has addressed this topic in the family Syngnathidae, but one study (Sária et al. 2013) and our anecdotal observations indicate that EE2 exposure does not cause males to adopt female-specific courtship behavior, which in Gulf pipefish includes a striking change in coloration, dancing and twitching (Partridge et al. 2013). Thus, the proximate factors involved in sex-specific mating behaviors in syngnathids remain unknown. As noted above (see Introduction), the waters are equally muddy for other sex-role-reversed taxa. In particular, testosterone, progesterone, estradiol, and prostaglandins have possible roles in modulating female behavior in various sex-role-reversed taxa (Eens and Pinxten 2000; Goymann et al. 2008; Gonçalves et al. 2014). The endocrinology of mating behavior in sex-role-reversed taxa should be a high priority

for research, as many interesting surprises no doubt await us, and syngnathid fishes provide a model uniquely suited to this research endeavor.

The pipefish liver transcriptome

In many ways, the Gulf pipefish liver transcriptome behaves in a similar fashion to those of the few other fish species studied in this regard. In control fish, pregnant and non-pregnant males shared more similar gene expression patterns than either group shared with females, and both groups of males responded in a female-like manner to synthetic estrogen. The most telling result is that genes involved in egg production are highly upregulated in females relative to males, a typical pattern in the livers of fish species with conventional sex roles. In addition, some of the most highly expressed genes in the livers of females also encode egg-related proteins and EE2 exposure induces expression of these genes in males.

However, at face value, our patterns of gene expression look quite different from those observed in zebrafish (Zheng et al. 2013), with only a few genes occurring on both species' top-50 lists with regard to expression levels. Similarly, only a handful of genes show similar, statistically significant responses to EE2 in both species. Nevertheless, the overall theme that egg-related protein genes are upregulated in females and that estrogen exposure feminizes male livers appears in both the Gulf pipefish and zebrafish datasets (Zheng et al. 2013). In addition, the zebrafish study identified a marked up-regulation of ribosomal genes in female livers, and while these genes did not appear on our list of extremely sexually dimorphic loci, many ribosomal genes did appear on our complete list of statistically significant sexually dimorphic genes (S1). Many of the differences

between our study and the zebrafish study could be a consequence of differences in experimental design. The zebrafish study used E2 rather than EE2, used a much higher concentration of sex hormones (1000-fold higher), pooled individuals within a treatment before sequencing, and provided some evidence that their males had already been exposed to endocrine disruptors before their use in the experiment (Zheng et al. 2013). Other studies involving zebrafish (Robison et al. 2008), yellow perch (Goetz et al. 2009), and turbot (Taboada et al. 2012) show patterns that are broadly consistent with our results, although the precise genes involved often differ considerably among datasets.

Another intriguing result from our analysis is that the low doses of EE2 used in our study resulted in masculinization of the female liver. This pattern is evident from our observation that 90 percent of sexually dimorphic genes showed expression changes in opposition to the pattern of sexual dimorphism in exposed females (i.e., their expression became more male-like). At face value, this observation might be interpreted as evidence that the female liver transcriptome is sex-role-reversed with respect to its response to estrogens. However, this sort of pattern has been observed in other taxa without sex-role reversal (Hoffmann et al. 2006; Colli-Dula et al. 2014), and the most likely explanation is simply that too much estrogen causes females to begin to shut down reproduction. Thus, this observation also seems to indicate that the pipefish liver is very much like that of other fish without sex-role reversal.

Sexual selection and the liver

One interesting possibility, which is hinted at by our data but not definitively supported by any of our analyses, is that the liver somehow plays a role in sexual

selection in sex-role-reversed pipefish. The female liver produces egg-related proteins, which are transported to the ovaries, processed and packaged in the eggs. In sex-role-reversed species, females typically have greater potential reproductive rates than males, which can result in pre- and postcopulatory competition among females for mating and reproductive success. Hence, female-specific genes in the liver controlling egg production could be under stronger sexual selection in females of sex-role-reversed species compared to related species with less mating competition among females, such as monogamous pipefishes and seahorses. Another plausible hypothesis is that other proteins produced by the liver are also transported to the female's reproductive tract and eventually transferred to the male during mating. Even though most studies of reproductive protein evolution focus on the gonads, the liver may be a neglected organ with an underappreciated role in postcopulatory processes. Interestingly, our data also show that some sperm-related proteins in males are produced in the liver, so males also could experience selection on genes expressed in the liver whose products are ultimately transported to the gonads or brood pouch. This idea remains speculative at the moment, but we feel that the role of the liver in postcopulatory processes, especially in sex-role-reversed organisms, is a topic that deserves further exploration.

Environmental endocrine disruptors in pipefish

Syngnathid fishes are potentially useful for the study of endocrine disruptors in marine environments, because many pipefishes and seahorses occur in shallow coastal waters throughout the world (Dawson 1985). Furthermore, many syngnathids, including the Gulf pipefish, lack a pelagic dispersal phase, so most individuals probably complete

their lifecycle near their birthplace. Thus, pipefish have the potential to serve as sentinel species for endocrine disruptors in the marine realm. Other work has shown that *vitellogenin* is upregulated in EE2-exposed pipefish males (Ueda et al. 2005; Sárria et al. 2013). Our results provide a number of other potential genes that could serve as biomarkers of environmental estrogens in male or female pipefish, although males seem to be the best choice for unambiguous evidence of endocrine disruption. One key feature of our study is that we used environmentally relevant concentrations of EE2 (5 ng/L). Studies of EE2 in contaminated bodies of water have revealed levels of EE2 in the range of 30-50 ng/L and even as high as 820 ng/L in some places (Ternes et al. 1999; Kolpin et al. 2002; Pojana et al. 2007). Hence, gene expression changes in pipefish livers could provide a sensitive method for the preliminary screening of sites with a high risk of deleterious impacts from endocrine-disrupting contaminants.

Conclusions

Our results provide an important advance in the endocrinology of sex-role-reversed vertebrates. In particular we found that the pipefish liver is sexually dimorphic and estrogen regulated, suggesting that female reproductive physiology is regulated by estrogens in pipefish in much the same way as in fish without sex-role reversal. In light of other work on sex-role-reversed taxa, primarily birds, this result suggests that the ancestral mechanism of hormonal control of female reproductive physiology has been retained across sex-role-reversed vertebrates. However, a survey of the literature shows that secondary sexual traits are controlled by a variety of mechanisms, and pipefish are somewhat unusual in that estrogens induce female ornaments in males. Finally, sex-

role-reversed mating behaviors remain a mystery, as their proximate modulation has not yet been resolved in any sex-role-reversed taxon. Our results also provide novel pipefish transcripts that respond to endocrine disruptors, which could be extremely useful for screening coastal marine habitats for environmental estrogens. In short, this study provides novel insights into sexual dimorphism and hormonal regulation of gene expression patterns in the pipefish liver, setting the stage for future studies regarding the endocrinology of sex-role-reversed mating behavior in this interesting marine fish.

5. GENETIC EVIDENCE FOR MONOGAMY IN THE DWARF SEAHORSE, *HIPPOCAMPUS ZOSTERAE*[*]

Introduction

The family Syngnathidae provides an excellent system in which to study variation in mating systems. From pair-bonding in monogamous seahorses to both polyandrous and polygynandrous pipefish species, the family Syngnathidae contains species with genetic mating systems at the extremes (Jones and Avise 2001; Wilson et al. 2003). The evolution of male pregnancy in syngnathid fishes has resulted in sex-role reversal in many species of pipefishes, in the sense that females compete for access to limited male brooding capacity, and sexual selection thus acts more strongly on females than on males. Seahorses, however, appear to have maintained conventional sex roles with males normally initiating courtship and more often interacting aggressively than females. Of the handful of seahorses that have been studied either socially or genetically, all appear to be monogamous (Jones et al. 1998; Wilson and Martin-Smith 2007; Mobley et al. 2011; Woodall et al. 2011). While seahorses exhibit monogamous pairing during single breeding events, males will often switch mates across breeding seasons in the European long-snouted seahorse, *H. guttulatus*, or even within a breeding season in the Western Australian seahorse, *H. subelongatus* (Kvarnemo et al. 2000, Woodall et al. 2011). Behavioral studies in the animal's natural environment or in the laboratory are both informative and necessary, but it is important to document genetic mating systems

[*] Reprinted with permission from Genetic evidence for monogamy in the dwarf seahorse, *Hippocampus zosterae* by Emily Rose, Clayton M. Small, Hector A. Saucedo, Cristin Harper, Adam G. Jones. 2014. *Journal of Heredity* 105:6. Copyright 2014 by Oxford University Press DOI: 10.1093/jhered/esu050

to understand fully the evolutionary consequences of the social mating system. A characterization of the genetic mating system is also a necessary prerequisite for more detailed comparative studies of the causes and consequences of mating system evolution within the Syngnathidae family.

The dwarf seahorse, *Hippocampus zosterae*, is of particular interest, because it is a common seahorse amenable to husbandry, making it an appealing model in comparative studies of mating behavior. Laboratory-based social experiments indicate that dwarf seahorses are monogamous, but this mating behavior has yet to be confirmed genetically (Masonjones and Lewis 1996). The dwarf seahorse is one of the smallest seahorse species, ranging from 16-38 mm in length, and can be found in shallow seagrass beds in the Gulf of Mexico and from the Bahamas to Bermuda (Masonjones and Lewis 1996; Strawn 1958). Like other species of seahorses, dwarf seahorse males have a sophisticated brood pouch, which is completely enclosed except for a small opening. This pouch configuration ensures paternity of offspring within the male's pouch (Jones et al. 1998; Jones and Avise 2001). While both male and female seahorses invest energy into the production of their offspring, the male invests much more energy than the female, as shown by a comparison of the metabolic rates of male and female dwarf seahorses (Masonjones 2001). Females make a non-trivial investment of energy in courtship and the generation of eggs, but males invest energy in courtship and in the carrying of the offspring over the entire pregnancy (Jones 2004). The courtship ritual for *H. zosterae* has four distinct phases with mating generally occurring on the third morning of courtship for unfamiliar pairs (Masonjones and Lewis 1996). The gestation

period for *H. zosterae* lasts for 13-14 days, after which re-mating will generally occur within a 4-20 hour window (Vincent 1994; Masonjones & Lewis 1996). Previous work focusing on seasonal demographic patterns of the dwarf seahorse have shown that 35 percent of males caught in Tampa Bay during the winter are pregnant and 65 percent of males collected from these sites during the summer are pregnant, indicating that they reproduce year round (Masonjones, unpublished data). Our first goal in the present study was to develop a panel of novel microsatellite markers polymorphic enough to determine parentage in *H. zosterae*. Our second goal was to infer parentage for broods of field-caught, pregnant males to test the hypothesis that the genetic mating system in natural dwarf seahorse populations is monogamy.

Methods

Two separate collecting sites were used to obtain samples of *H. zosterae* from the Gulf of Mexico. Pregnant males were collected and sacrificed in Tampa Bay, Florida during a single day of seining on August 24th, 2009. The second sample of pregnant males was collected over four separate days of seining (July 14th, August 17th, August 28th, and September 27th) during 2010 in Redfish Bay, Texas. Males collected in Texas were sacrificed on the day of collection if the developing embryos were more than halfway through their two-week gestation period, or sacrificed after allowing them to give birth in laboratory tanks if the embryos were in early stages upon collection. This approach ensured the offspring were large enough for genotyping using Chelex extractions. We sacrificed all adult male fish using an overdose of MS222 and preserved tissue clippings from the seahorses' tails in 95% ethanol. The DNA from the adult tissue

samples was extracted using the Genomic DNA Purification Kit from Gentra systems (Qiagen) and diluted to a concentration of 50 ng/µL. The paternity of each fry was known as a result of the fry being individually dissected from the father's pouch or collected from the tank in which an isolated male gave birth. For the DNA extraction of the offspring, each fry was placed in 150 µl of Chelex and Proteinase K solution (stock: 25 ml millipure water, 199 µl Proteinase K [20mg/ml] and 1.25g Chelex) and incubated at 56 ºC for one hour followed by 100 ºC for eight minutes.

We developed a panel of hypervariable microsatellites, ultimately using four (*Hzos04*, *Hzos05*, *Hzos06*, and *Hzos 07*) to determine maternity for each of the males' broods (Table 9). We isolated the markers from a single, Texas-caught adult using a protocol originally developed by Ardren et al. (2002), but modified in exact accordance with Mobley et al. (2009). In short, we produced a library from genomic DNA enriched for GATA repeats by degenerate oligonucleotide-primed PCR fragmentation (Macas et al. 1996), followed by hybridization to a biotinylated $(GATA)_8$ probe, streptavidin bead pull-down, elution, and cloning with the Topo TA Cloning Kit (Invitrogen). Forty-four of 576 colonies screened positively for a putative repetitive insert, and we sequenced these clones in both directions at the Nevada Genomics Center (Reno, NV) using T3 and T7 primers. Eight of these clones proved to be unique microsatellite sequences amenable to PCR primer design, which we performed with the assistance of Primer3 version 0.4.0 (Rozen and Skaletsky 2000) and default settings. Table 9 provides relevant details for the eight markers. Markers *Hzos04*, *Hzos05*, *Hzos06*, and *Hzos07* were consistently

amplifiable based on PCR optimization assays and proved to be polymorphic, so we used these for all subsequent parentage analyses.

Each of the microsatellites was amplified in 25 µL reactions containing 17.25 µL purified water, 2 µL $MgCl_2$ (25nM), 1 µL deoxynucleotide triphosphate mix (2mM dNTPs), 2.5 µL 10X PCR buffer, 0.5 µL forward (5' fluorescently labeled) and reverse primers (10µM), 0.25 µL Taq polymerase, and lastly 1 µL of template DNA (50ng/ µL). Thermal cycling consisted of five steps with the initial denaturing step occurring during the first two minutes at 94°C for all four microsatellite loci. Next followed 39 cycles for *Hzos*04, *Hzos*06, and *Hzos*07 and 31 cycles for *Hzos*05 including one minute at 92°C for denaturing, annealing temperatures of 60°C for one minute for *Hzos*06 and *Hzos*07, 62°C for one minute for *Hzos*04, and 60.7°C for 50 seconds for *Hzos*05 and lastly, extension at 72°C for one minute. Each PCR concluded at 72°C for four minutes, followed by storage at 4°C. Products were confirmed on 2% agarose gels and analyzed on an Applied BioSystems 3730xl DNA Analyzer at the Cornell University Life Sciences Core Laboratories Center. The alleles for all four loci were sized using Peak Scanner software (Applied Biosystems).

Table 9 Microsatellite structures, primers, accession numbers, annealing temperatures and total number of alleles for eight unique microsatellite markers developed for *H. zosterae*.

Locus	Structure	F Primer Sequence (5'-3')	R Primer Sequence (5'-3')	Accession #	Amplifies?	Polymorphic?
Hzos01	$[GATA]_{19-34}$	AATATGAAAAATTATTGGAAGATACTG	AGTTGAGGGTATTTGCAGGA	KJ159958	Yes	N/A
Hzos02	$[TCTA]_3[GATA]_{29}$	GACTTCATGAACCACCGAAAC	AATCCGATTGATTCACGTTTG	KJ159959	Yes	N/A
Hzos03	$[GATA]_{16-27}$	CACTGACGTACTTGTTGCTGTG	ACCCGAGGGGTGAGACAAG	KJ159960	Yes	N/A
Hzos04	$[GATA]_{33-36}$	TCGTGATTTTAATGCCAATCC	TTATGATGATCCAGTTTGCTTTC	KJ159961	Yes	Yes
Hzos05	$[GATA]_{96}$	GCACAGCGACTCTGGATAAG	GGATCATCTGGCATTTCAGC	KJ159962	Yes	Yes
Hzos06	$[GATA]_{12}$	CGAATGAAATATTTTGGGGAAC	TGAAATTCCGTGCTAAAAAGC	KJ159963	Yes	Yes
Hzos07	$[GACA]_8[GATA]_{29}$	ATGGTACCAAGCCCAAACTG	ACCCATGAAAAGTTGGAACG	KJ159964	Yes	Yes
Hzos08	$[GATA]_{47}$	TGGCAAAAAGTTTGGACACC	TGAAAATTCAAGCAAAATGTCAG	KJ159965	Yes	N/A

We first reconstructed the parental genotypes for every offspring within a brood and determined either the maternal genotype or both parental genotypes (in some cases the father's tissue sample did not produce amplifiable DNA) using GERUD 2.0 (Jones 2005). Next, we calculated exclusion probabilities for both a single known parent and both unknown parents for Texas and Florida separately by using GERUD 2.0 (Jones 2005). We determined the power of detecting multiple maternity using the program GERUDsim 2.0 (Jones 2005) to test the probability of identifying the correct number of sires in simulations with various ratios of maternal contributions using the adult genotypes. Simulations were run using the "known mother" setting, where the program assumes one of the parental genotypes is known, because the paternity of our fish's offspring is known. We ran 1,000 simulations with either equal (50:50) or skewed (25:75) maternal contributions to broods using the study's average brood size of 16. We also summarized genetic differentiation between adults in each of the populations to

determine F_{ST} values and tested for linkage disequilibrium among loci using Arlequin 3.5 (Excoffier and Lischer 1995). Finally, we used GENEPOP to test if the loci in each population deviated from Hardy-Weinberg equilibrium and determine the level of expected heterozygosity, H_E (Raymond and Rousset 1995). In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses with Dryad.

Results

We genotyped broods from a total of 16 pregnant males, resulting in 248 embryos assayed for an average of 15.5 offspring per male. Within each male's progeny array, we detected a maximum of four alleles at each locus (Table 10). As a result, all 16 broods were confirmed to have a single maternal genotype when analyzed with GERUD 2.0 (Table 10). A single null (non-amplifying) allele appeared in one of the male seahorses, FL-M12, and his offspring. This null allele was detected during the parentage assignment using GERUD 2.0 and the male was identified as a null heterozygote rather than a normal homozygous individual (Jones and Ardren 2003). All of the loci were highly polymorphic with 27-36 alleles represented across the 32 parental fish genotypes, which include the 16 genotypes of wild-caught, pregnant males and the inferred genotypes of the 16 females assigned as their mates (Table 9).

Table 10 Brood sizes, allele numbers per brood, and maximum number of mothers for each male collected in Texas (TX) and Florida (FL).

Loci	Number of Alleles		H_E		Exclusion Probability			
					Neither Parent Known		One Parent Known	
	TX	FL	TX	FL	TX	FL	TX	FL
Hzos04	19	19	0.97	0.97	0.76	0.78	0.86	0.87
Hzos05	20	23	0.99	0.97	0.82	0.78	0.90	0.88
Hzos06	20	22	0.98	0.97	0.80	0.78	0.89	0.88
Hzos07	17	18	0.95	0.95	0.73	0.75	0.84	0.86

The four microsatellites we used for this particular study were polymorphic enough to easily identify the maternal contribution to each male's brood, allowing us to use only half of the eight markers we developed. Although the samples sizes for each population were small, we found no evidence of significant genetic differentiation between the two populations (Fst: 0.00, $p = 0.354$), and none of the loci were found to be in linkage disequilibrium ($p = 1.00$) (Table 11). There also were no major differences between the adults within the Florida and Texas populations in ranges of expected heterozygosity (0.95-0.99), and none of our loci deviated from Hardy-Weinberg equilibrium in either population ($p > 0.05$) (Table 11). Simulations run using GERUDsim2.0 determined that the probability of detecting multiple mating was 99.7 and 100 percent in Florida and Texas, respectively, in cases with skewed maternal contributions (25:75) and 99.8 and 99.8 percent assuming even maternal contributions. Thus, these markers were sufficiently polymorphic that we would have detected multiple mating by males had it occurred in our sample.

Table 11 Summary statistics for the four microsatellites used to assign parentage. Including the total number of alleles, the levels of expected heterozygosity (H_E), and exclusion probabilities calculated with one parent known and neither parent known for all individuals in the two populations collected in Texas (TX) and Florida (FL).

Loci	Number of Alleles		HE		Exclusion Probability			
					Neither Parent Known		One Parent Known	
	TX	FL	TX	FL	TX	FL	TX	FL
Hzos04	19	19	0.97	0.97	0.76	0.78	0.86	0.87
Hzos05	20	23	0.99	0.97	0.82	0.78	0.90	0.88
Hzos06	20	22	0.98	0.97	0.80	0.75	0.89	0.86
Hzos07	17	18	0.95	0.95	0.73	0.75	0.84	0.86

Discussion

The eight microsatellite markers produced by our study should serve as useful resources for researchers wishing to embark on future molecular ecological studies of the dwarf seahorse. The four markers we used to infer parentage in this study have proven their worth in this context, and all eight microsatellites could in principle be applied fruitfully to basic studies of genetic structure among dwarf seahorse populations, or even those of other seahorse species. The results from the parentage analyses of the males' broods from both collecting locations, Florida and Texas, indicate that males mate singly, maternal genotypes are unique to each brood, and therefore dwarf seahorses are indeed genetically monogamous. These results support previous findings from behavior trials in the laboratory where this particular species of seahorse was shown to exhibit a socially monogamous mating system (Masonjones and Lewis 1996). To date, all species of seahorses that have been tested using microsatellite-based parentage

analyses, including *H. abdominalis*, *H. subelongatus* (formerly known as *H. angustus*), *H. guttalatus* and now *H. zosterae,* have been shown to be genetically monogamous (Jones et al. 1998; Wilson and Martin-Smith 2007; Woodall et al. 2011). Similar to other monogamous species, seahorses have little variation in morphology between sexes compared to the sexually dimorphic and ornamented pipefish species (Mobley et al 2011). One possible explanation for the maintenance of monogamy in seahorses is that the female seahorse produces her eggs in discreet batches and must transfer the entire batch to a male before preparing subsequent batches (Vincent 1994; Jones and Avise 2001). Some pipefish species, on the other hand, continuously produce mature eggs and can transfer subsets of these eggs to different males in rapid succession (Kornienko 2001). As a result of this physiological constraint in egg production, it becomes wasteful for a seahorse female to invest energy in the production of eggs unless she can secure a willing male to receive them. Whether this egg maturation process evolved before or after the evolution of monogamy in the seahorse lineage remains an open question.

Another possible explanation for monogamy in seahorses is that low population densities increase the cost of searching for additional mates (Emlen and Oring 1977), a situation that is usually thought to favor the formation of pair bonds. However, not all seahorses have low population densities, as previous studies on *H. guttulatus* have shown population densities to be high enough that each individual would have access to many prospective mates during the breeding season (Curtis and Vincent 2006). Moreover, there is still little known about the ecological and demographic setting in

which the most recent common ancestor of seahorses evolved, likely more than 20 million years ago (Casey et al. 2004, Teske et al. 2004). It is suggested that the genus *Hippocampus* originated in the West Pacific region with the most basal species of the genus inhabiting Australia (Teske et al. 2004, Wilson and Orr 2011). Phylogenetic reconstructions also estimate the divergence time between seahorses and their nearest extant relatives, the pygmy pipehorses of genus *Idiotrpiscis*, to be 25 to 28 Myr, indicating the upright posture of seahorses evolved during the Late Oligocene (Wilson et al. 2001, Teske et al. 2009).

While monogamy seems to be the norm for all *Hippocampus* species studied thus far, other syngnathid genera display a wide range of mating systems. Mating system variation within family Syngnathidae ranges from social monogamy in seahorses and the pipefish *Corythoichthys haematopterus,* to polygynandry in *Syngnathus floridae, S. typhle, S. leptorhynchus,* and *S. abaster,* and polyandry in *Nerophis ophidion* and *S. scovelli* (Hübner et al. 2013; Jones and Avise 1997*a*,*b*; Jones et al. 1999; Matsumoto and Yanagisawa 2001; McCoy et al. 2001; Wilson 2009). This variation in syngnathid mating systems, particularly within the genus *Syngnathus*, underscores the notion that we should be cautious in drawing generalizations regarding an entire genus from observations involving one or a few species. However, our genetic data do add additional support to the hypothesis that all seahorses in the genus *Hippocampus* retain a monogamous mating system that was likely present in their most recent common ancestor. In conclusion, our results confirm genetically the previous behavioral studies documenting the dwarf seahorse as a monogamously mating species, and also provide

new, highly polymorphic microsatellites to utilize for future studies regarding this diminutive seahorse species.

6. CONCLUSIONS

Section summaries

The main goal of the second section of my dissertation was to address the contributions of premating and postmating selection episodes towards total selection in a sex-role-reversed pipefish. Empirical studies of sexual selection often focus on events occurring either before or after mating but rarely both and consequently may fail to discern the relative magnitudes and interactions of premating and postmating episodes of selection. To address the main goal of the second section, premating and postmating selection was simultaneously quantified in the sex-role-reversed Gulf pipefish by using a microsatellite-based analysis of parentage in experimental populations. Female pipefish exhibited an opportunity for selection (***I***) of 1.64, which was higher than that of males (0.35). Decompositions of ***I*** and the selection differential on body size showed that over 95 percent of the selection on females arose from the premating phase. The results in this section also provided evidence for a tradeoff between selection phases, where multiply mating females had significantly lower offspring survivorship compared to singly mated females. In males, variance in relative fitness arose mainly from the number of eggs received per copulation and a small number of males who failed to mate. Overall, the second section of my dissertation exemplifies a general approach for the decomposition of total selection into premating and postmating phases to understand the interplay among components of natural and sexual selection that conspire to shape sexually selected traits.

The third section of my dissertation focuses on the effects of synthetic estrogen exposure on total selection in the sex-role-reversed Gulf pipefish. Environmental estrogens have been shown to affect populations of aquatic organisms in devastating ways, including feminization of males, alterations in mating behaviors, and disruption of sexual selection. Studies have shown 17α-ethinylestradiol (EE2) exposure to induce female-like secondary sexual traits in male Gulf pipefish, changing how females perceive affected males. To determine the effects of EE2 exposure on the sex-role-reversed mating system and the strength of selection artificial Gulf pipefish breeding aggregations were set up in the laboratory at Texas A&M University. A microsatellite-based parentage analysis was to determine maternity of the offspring and the opportunity for selection and selection differentials on body size for both sexes were calculated for three consecutive episodes of selection for both the control and EE2 exposed replicates. Exposure to EE2 did not affect the strength of selection, likely due to the unusual sex-role-reversed mating system found in this species. With respect to multiply mated females, EE2 exposed females produced more eggs with higher embryo survivorship than non-exposed females. Thus, short-term exposure to low concentrations (2.0 ng/L) of EE2 in Gulf pipefish enhanced female reproductive success. However, higher EE2 concentrations (5.0 ng/L) caused complete reproductive failure in Gulf pipefish males. The results from the third section ultimately shed light on the need for studies addressing the long-term effects of EE2 exposure in Gulf pipefish in both artificial and natural populations.

The fourth section of my dissertation investigated gene expression patterns in the liver of the sex-role-reversed Gulf pipefish, because the liver is known to be sexually dimorphic and estrogen-regulated in species with conventional sex roles. Using next-generation RNA-sequencing technology (RNA-seq), sexually dimorphic hepatic gene expression patterns were detected, with a total of 482 differentially expressed genes between the sexes in Gulf pipefish. Two-thirds of these genes were over-expressed in females, and the sex-specific transcriptomes of this sex-role-reversed pipefish's liver were superficially similar to those of fishes with conventional sex-roles. Females, pregnant males, and non-pregnant males were exposed to 17α-ethinylestradiol (EE2) at ecologically relevant concentrations of 5ng/L and compared gene expression patterns in the livers of exposed fish to control fish. Several genes that were up-regulated in EE2-exposed males relative to control males were also found to be female-biased in control animals. These genes included several of the classic estrogen biomarkers, such as *vitellogenin*, *choriogenin*, and *zona pellucida*. Thus, estrogen exposure induced feminization of the male liver transcriptome in a sex-role-reversed pipefish. These results suggest that the ancestral state of estrogen-regulated female reproductive physiology has been retained in all sex-role-reversed vertebrates thus far studied, despite substantial evolution of the hormonal regulation of ornamentation and mating behavior in these interesting taxa.

The focus of the fifth section of my dissertation was to address if the dwarf seahorse, *Hippocampus zosterae*, is genetically monogamous. Syngnathid fishes (pipefishes, seahorses and seadragons) exhibit a wide array of mating systems ranging

from monogamy with long-term pair bonds to more promiscuous mating systems, such as polyandry and polygynandry. Some seahorses, including the dwarf seahorse *Hippocampus zosterae*, have been found to be socially monogamous. While several seahorse species have also been shown to be genetically monogamous, parentage analysis has not yet been applied to the dwarf seahorse. Eight novel microsatellites for the dwarf seahorse were developed to conduct a microsatellite-based parentage analysis to confirm that this species was indeed monogamous. Using a total of sixteen pregnant male seahorses, with eight collected in Florida and eight sampled in Texas, all of the offspring within a male's brood were genotyped to determine the maternal contributions to each brood. The results showed a maximum of four alleles per locus segregating within each pregnant male's brood, a pattern consistent with each brood having exactly one mother and one father. These results support previous laboratory-based behavioral studies and indicate that the dwarf seahorse, *H. zosterae*, is genetically monogamous.

Final conclusions

Species exhibiting sex-role reversal provide an unusual perspective on the evolution of sex roles and sex differences. However, the proximate effects of sex-role reversal are largely unknown. Endocrine disruptors provide an experimental mechanism to address hormonal regulation of sexually dimorphic gene expression in sex-role-reversed taxa. Previous studies have shown that synthetic estrogen, EE2, exposure can feminize the morphology of sex-role reversed Gulf pipefish males at relatively high concentrations of 100 ng/L EE2 (Partridge et al. 2010). At low, ecologically relevant EE2 concentrations of 2ng/L, the non-exposed females who mated multiply in the

control treatments transferred fewer eggs than singly mated females, but this is not the case for EE2 exposed females. There was no evidence of a tradeoff between the number of mates and the amount of eggs a female transferred in the EE2 treatment and unlike their control counterparts, the EE2 exposed multiply mated females were not limited in the number of eggs to transfer to their mates. These results indicated a hormesis effect due to the low levels of EE2 (2 ng/L) exposure resulting in exposed females having greater reproductive successes, leading to increased sexual selection acting on exposed females. The findings of my first two publications also revealed that in both exposed and non-exposed Gulf pipefish treatments, selection acts more strongly on females, with the majority of the variance in female fitness resulting from the first episode of selection and indicating there was a large amount of variance in female mating success. In addition, the results from this dissertation show that the understanding the consequences from exposure to different concentrations of EE2 are important because at slightly higher, yet ecologically relevant concentrations of EE2 (5 ng/L), there was morphological feminization and complete reproductive failure in exposed male pipefish, even though these concentrations still allowed for increased egg production in exposed females.

Using next-generation sequencing techniques has enabled me to test several questions regarding the effects of synthetic estrogen exposure in male and female Gulf pipefish. By using RNA-sequencing technology, I was able to identify genes with sex-specific expression patterns and test the impacts of EE2 exposure on the liver transcriptomes of exposed Gulf pipefish. This study was the first to indicate that pipefish have sexually dimorphic liver gene expression patterns which are similar to other fish

that possess conventional sex-roles in terms of their physiology, even though this species is considered to exhibit behavioral sex-role-reversal. In regards to the effects of EE2 exposure on gene expression patterns, I determined that low, ecologically relevant levels of synthetic estrogen exposure caused feminization of the exposed male liver transcriptomes, with a greater effect on the livers of pregnant males, and had the reverse effect on exposed female fish. This in turn meant that each sex responded differently to the estrogen exposure due to their reproductive physiology. I was also able to identify potential genetic biomarkers of estrogen exposure from the results of the RNA-sequencing of the liver transcriptomes, which can be used as candidate genes to detect exposure to synthetic estrogen or chemicals with estrogenic modes of action in wild populations of fish.

There are several future directions for this project, including studies in the laboratory and in natural populations. The next step is to identify the effects of EE2 exposure on male reproductive abilities and identify the genes that have altered expression patterns in the pipefish male's brood pouch of both pregnant and non-pregnant males when exposed to ecologically relevant concentrations of EE2. Thus far, I have identified gene expression pattern changes in the brood pouches of exposed pregnant and non-pregnant males and found that exposure to EE2 appears to have a much smaller effect on males when they are exposed after their broods have been received and fertilized compared to the males exposed prior to mating, which often resulting in reproductive failure. These results, paired with the results of my third section of the dissertation, suggest that the timing and concentration of the EE2 exposure plays a

large role in the evolutionary and reproductive impacts on these unique fish. Lastly, the final direction for this project would be to monitor and assess the impacts of EE2 exposure in naturally exposed populations by using the biomarkers that were identified from the RNA-sequencing results. However, there are many difficulties in addressing this question outside of the laboratory setting because in the natural environment fish are exposed to a combination of contaminants, including chemicals with estrogenic, androgenic or anti-androgenic effects, which can all interact with the physiology of the exposed individuals, making it difficult to isolate the effects of a single contaminant.

Aquatic organisms are especially vulnerable to endocrine disruptor contamination because of direct contact with the contaminated environment because of the constant interaction of the contaminants during development and also because determination of sex can be sensitive to hormonal contaminants (Orlando and Guillette, 2007). The results of this dissertation have shown that understanding the effects of endocrine disruptors at various concentrations is a key step in predicting the impacts in natural populations after exposure to synthetic estrogen. While increased feminization of males resulting from EE2 contamination has been well documented, it is now evident from the results present in this dissertation that the effects of these contaminants are altering exposed organisms at the genomic level at low concentrations, before any morphological changes occur. The findings of this dissertation are important because exposure to low, ecologically relevant concentrations of endocrine disruptors could have drastic effects on their reproductive abilities, recruitment, social behaviors, and gene expression patterns in natural populations.

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APPENDIX

Supporting information titles

S1: Full list of differentially expressed genes between control females and control males

S2: Full list of differentially expressed genes between control pregnant males and control non-pregnant males

S3: Full list of differentially expressed genes for EE2 exposed pregnant males versus control pregnant males

S4: Full list of differentially expressed genes for EE2 exposed non-pregnant males versus control non-pregnant males

S5: Full list of differentially expressed genes for EE2 exposed pregnant males versus EE2 exposed non-pregnant males

S6: Full list of differentially expressed genes for EE2 exposed females versus control females

S7: A PCA for all six treatments using all genes with a mean number of transcripts equal or greater than 3,090. Although the treatments of the same sex group together, EE2 exposed pregnant males cluster closer to the female treatments than other male groups.